Amino acid nutrition and immune function in tumour-bearing rats: a comparison of glutamine-, arginine- and ornithine 2-oxoglutarate-supplemented diets

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ABSTRACT

Dietary supplementation with glutamine (Gln), arginine (Arg) or ornithine 2-oxoglutarate (α-ketoglutarate; OKG) has attracted recent attention for the potential to improve anti-cancer immune function. However, since these compounds have not been compared systematically in an internally controlled study, their relative efficacy is difficult to estimate. Buffalo rats were fed on nutritionally complete semi-purified diets supplemented with Gln, Arg or OKG for 14 days after implantation of the Morris hepatoma 7777 (n = 7 per diet). The control diet was made isonitrogenous and isoenergetic by addition of a mixture of non-essential amino acids. After 14 days, peritoneal macrophages and splenocytes were isolated to determine cell phenotypes, macrophage cytostatic activity and natural killer (NK) cell cytotoxicity, as well as nitric oxide (NO) and cytokine production. Diet had no effect on tumour weight (1.6 ± 0.2 g; n = 59). However, rats fed OKG had increased macrophage cytostatic activity and NK cell cytotoxicity (P < 0.05). Although enhanced killing ability by NK cells was associated with higher splenocyte NO production (P < 0.04), increased cytotoxicity was not inhibited by a specific inhibitor of inducible NO synthase. The proportion of interleukin-2-receptor-positive T cells after stimulation increased in rats fed OKG (P < 0.05); however, cytokine production was not affected by diet. None of OKG, Gln or Arg altered tumour growth compared with a control mixture of non-essential amino acids. These results suggest no net advantage for anti-cancer immunity, but do not preclude benefits in immune responses to disease recurrence or metastasis, therapy or secondary infection.

INTRODUCTION

Anti-cancer immunity consists, in part, of a cellular component involving macrophages and natural killer (NK) cells [1,2]. There are many putative mechanisms of cytotoxicity by immune cells, including production of interleukin-2 (IL-2) by CD4+ Th1 cells, which activates both macrophages [3] and NK cells [2]. Interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and nitric oxide (NO), produced by various activated immune cells,
have also been identified as key molecules in anti-cancer defence [4–6]. Since anti-cancer immunity declines progressively with tumour growth [7], an important clinical goal is identifying means of stimulating host immunity. Recently, pharmaconutrients such as glutamine (Gln), arginine (Arg) and ornithine 2-oxoglutarate (α-keto-glutarate; OKG) have attracted attention for their potential immunoenhancing properties.

Since Gln is an essential fuel for rapidly dividing immune cells [8], Gln-depleted states are associated with impaired immune function [9]. Studies of Gln supplementation in tumour-bearing animals suggest improvement of anti-cancer immune defences of T cells [10], NK cells [11] and lymphokine-activated killer cells [12]. Although Gln is the primary amino acid utilized by most rapidly proliferating tumours [13], Gln supplementation has been shown to have no effect on or to inhibit tumour growth in animals [10,14,15].

An Arg requirement for optimal lymphocyte proliferation, T cell and macrophage function, and cytokine and NO production has been reported [16]. Arg has been demonstrated to decrease the growth of experimental tumours [17,18], an effect which may be mediated through the immune system [19]. However, supplemental Arg can augment tumour growth in some animal models and human tumours [20–23].

OKG has been proposed for the treatment of the effects of surgery, burn, sepsis and cancer [24]. Potential mechanisms of action of OKG include the synthesis of several key metabolites that become depleted in stress situations, including Gln, Arg and proline [24]. Since OKG may be metabolized to form Gln, Arg and polyamines, it clearly has potential as an immuno-enhancing nutrient [24–26]. Enteral OKG has been shown to have no effect on tumour growth in rats [27,28]. In situations of stress, including cancer, trauma, burns and sepsis, amino acids previously considered to be non-essential may become indispensable [24]. The demonstrated requirements for Arg, Gln and their precursors/metabolites (ornithine and 2-oxoglutarate) are related, at least in part, to their involvement in cell proliferation and immune responses. In theory, these nutrients are metabolically interconvertible by known pathways, each potentially being a biosynthetic precursor of the others. Since it is not clear which of Arg, Gln and OKG is most critical to anti-tumour defence, and since they have not been compared systematically with one another in an internally controlled study, their relative efficacy is difficult to estimate. An additional difficulty in interpreting the current literature is that dietary amino acid supplementation has been studied over a wide range of cancer models and tumour burdens, ranging in size from < 1% to 30% of host body weight (e.g. [10,20, 28–30]). The present study was designed to compare the relative efficacy of dietary Gln, Arg and OKG in altering plasma and tissue amino acid profiles, tumour growth and host immune defence in rats bearing a tumour burden equivalent to ~ 0.8% of body weight and showing depletion of intracellular Gln, Arg and ornithine pools.

**MATERIALS AND METHODS**

**Materials**

RPMI 1640 culture medium, Dulbecco’s minimal essential medium, fetal calf serum, antimycotic/antibiotic solution (penicillin (0.1 i.u/ml), streptomycin (0.1 μg/ml) and amphotericin B (25 mg/l)) and Hepes were purchased from Gibco (Burlington, ON, Canada). Arg, Gln, BSA, 2-mercaptoethanol, lipopolysaccharide (LPS), ionomycin and Griess reagent chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concanavalin A (Con A), phorbole 12-myristate 13-acetate (PMA) and Ecolite® scintillation fluid were purchased from ICN (Montreal, QB, Canada). The OX19, w3/25, OX8, OX12 and OX42 antibodies were kindly provided by A. Rabinovitch (University of Alberta, Edmonton, Alberta, Canada). All monoclonal antibodies were mouse anti-(rat IgG). Phycoerythrin-conjugated goat anti-(mouse IgG) and all other antibodies (except J319) were purchased from Cedarlane Laboratories Ltd. (Hornby, ON, Canada). Fluorescein isothiocyanate-conjugated goat anti-(mouse IgG) was obtained from Organon Teknika Inc. (Scarborough, ON, Canada). Antibody J319 and IL-2 assay components were obtained from PharMingen (Mississauga, ON, Canada). A macrophage-sensitive murine mastocytoma cell line (P815) and an NK-cell-sensitive murine lymphoma cell line (YAC-1) were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Na²⁶⁰⁰Cr and [methyl-H]thymidine were obtained from Amersham (Oakville, ON, Canada).

**Animals and dietary design**

Animal studies were reviewed and approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee, and were conducted in accordance with the Canadian Council on Animal Care guidelines. Female rats of the Buffalo strain were obtained from a colony maintained at the University of Alberta, and were housed in individual cages, in a temperature- (24 °C) and humidity- (80%) controlled room. After a 7 day adaptation period, rats were randomly allocated to serve as healthy controls (n = 16) or were implanted with the Morris hepatoma 7777 (n = 64) as previously described [28]. Briefly, rats were anaesthetized with halothane, and 25 μl of finely chopped Morris hepatoma 7777 cells from a single donor animal were implanted subcutaneously in one flank.

Rats were randomly allocated to one of five treatments: (1) healthy controls fed a control mixture of amino acids,
Amino acid nutrition and immunity in cancer

Table 1 Composition of experimental diets

Diets were formulated and prepared in the laboratory, and were isoenergetic (gross energy 15.48 MJ/kg of diet) and isonitrogenous (15.8 g of nitrogen/100 g; 26.1% crude protein, inclusive of the amino acid supplements). The Gln-, Arg- and OKG-supplemented diets contained 0.28 mol of Gln, Arg or ornithine/kg of diet respectively. The control amino acid mixture was an isomolar mixture of alanine, glycine, serine and histidine. The fat mixture contained 2% linseed oil, 39.2% hard beef tallow and 58.8% safflower oil, providing a polyunsaturated/saturated fatty acid ratio of ~0.9. All rats were given free access to the experimental diet and water for 14 days after tumour implantation.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/kg of diet)</th>
<th>Ingredient</th>
<th>Control</th>
<th>Gln</th>
<th>Arg</th>
<th>OKG</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Gln</td>
<td>—</td>
<td>40</td>
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<td>—</td>
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<tr>
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<td>OKG</td>
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<td>—</td>
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</tbody>
</table>

* American Institute of Nutrition 76 (ICN Biochemicals, Cleveland, OH, U.S.A.).

(2) tumour-bearing rats fed a control mixture of amino acids, (3) tumour-bearing rats fed a diet supplemented with Arg, (4) tumour-bearing rats fed a diet supplemented with Gln, and (5) tumour-bearing rats fed a diet supplemented with OKG (Table 1). All diets met or exceeded the requirements of growing rats as specified by the National Research Council [31]. Semi-purified diets were formulated and prepared in the laboratory to be isoenergetic (gross energy 15.48 MJ/kg of diet) and isonitrogenous (26.1% crude protein, inclusive of the amino acid supplements described below). A constant portion of the diet (900 g/kg of diet) was based on casein, cornstarch and dextrose, and contained 92% and 63% of the total dietary energy and protein respectively. The constant portion of the diet also contained 200 g of fat from a mixture of sources (2% linseed oil, 39.2% hard beef tallow and 58.8% safflower oil), providing a polyunsaturated/saturated fatty acid ratio of ~0.9 [32]. A variable portion of the diet (100 g/kg of diet) contained Gln (4.0%, w/w), Arg (4.9%, w/w), OKG (5.8%, w/w) or a control mixture of amino acids. The control amino acid diet consisted of an isomolar mixture of four amino acids (alanine, glycine, serine and histidine) which have limited or no metabolic interaction with Gln, Arg and ornithine and are not known to be limiting for growth or immune function in rats of this age. The diets were isomolar for the nitrogen-containing constituent (0.28 mol of Arg, Gln or ornithine per kg of diet). The variable portion of each diet was made isonitrogenous and isoenergetic by the addition of the control amino acid mixture and/or cornstarch.

From day 0 to day 14, rats consumed 2.7±0.1, 3.2±0.1 and 3.7±0.2 g·kg⁻¹·day⁻¹ of Gln, Arg and OKG respectively. All rats were given free access to water and the experimental powder (not pelleted) diet for 14 days after tumour implantation. At the end of this period, rats were killed by CO₂ asphyxiation and cervical dislocation, followed by collection of the spleen, peritoneal macrophages and tibialis anterior muscle. In a subset of animals on each treatment (n = 7 per group), blood was collected under halothane anaesthesia by cardiac puncture for amino acid analysis. Due to the number of variables being measured, this study was carried out in a series of replicate experiments, such that all measurements were not performed on individual animals. For each assay, the number of animals in each treatment group is provided in the text, table or legend.

Plasma and skeletal muscle amino acids

Muscle and plasma free amino acid fractions were prepared as previously described [27] and were separated by HPLC using a Varian 5000 HPLC instrument (Varian,
Palo Alto, CA, U.S.A.) with a fluorichrom detector, using o-phthaldehyde as the fluorescent reagent. The column used was a Supelcosil 3 μm LC-18 reverse-phase column (4.6 mm × 150 mm; Supelco, Bellafonte, CA, U.S.A.). Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom chromatography data system (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.).

Immune variables
A number of different tumour models in various inbred strains of rats are used for studying anti-tumour immune defence [33]. Our model is the Morris hepatoma 7777, a well characterized, poorly differentiated and rapidly growing transplantable tumour. Tumor excision–rechallenge studies by Wepsic et al. [34] demonstrated the presence of tumour antigens in the Morris hepatoma 7777 cell line. Previous work in our laboratory demonstrated a progressive suppressive effect of this tumour on components of both natural and acquired anti-tumour immunity [7]. We have also previously shown that the Morris hepatoma 7777 captures a significant amount of the host’s amino acid intake for protein synthesis and oxidation [28]. Reduced tissue pools of Gln, glutamate and Arg during growth of the Morris hepatoma 7777 [35] may be associated with immune suppression. To measure anti-tumour immune defence, we used several well characterized ex vivo immune assays as substitutes for in vivo immune assessment. Standard target cell lines (e.g. YAC-1 and P815) are used extensively by immunological researchers to assess immune cell cytotoxicity or cytostasis in both humans and animals. Their use facilitates comparison of our results with those in the published literature.

Peritoneal macrophage isolation and culture
Resident peritoneal macrophages were obtained by sterile peritoneal lavage using cold RPMI 1640 containing glutamine (300 mg/l) and 1% (v/v) antimycotic/antibiotic solution, pH 7.4, and cell viability was assessed by Trypan Blue exclusion. Macrophages were washed by centrifugation at 380 g for 5 min at 4 °C and resuspended (4 × 10^6 cells/l) in the appropriate volume of macrophage complete culture medium [RPMI 1640 containing 4% (v/v) heat-inactivated fetal calf serum, glutamine (300 mg/l), 1% (v/v) antimycotic/antibiotic solution and 2-mercaptoethanol (2.5 μmol/l)], pH 7.4. Macrophages (0.8 × 10^6/well) were incubated in sterile 96-well round-bottom microtitre plates for 2 h in a humidified atmosphere at 37 °C in the presence of 5% CO_2 to allow for macrophage adherence. After 2 h, wells were washed three times in 200 μl of sterile Krebs–Ringer/Hepes buffer (pH 7.4) at room temperature to remove non-adherent cells. The purity of the isolated macrophage population was assessed by indirect immunofluorescence assay, as described below. Preliminary experiments showed that maximal stimulation of macrophages occurred using 10 μg/ml LPS from Escherichia coli (Serotype O55:B5) for 24 h (results not shown). Thus, for determination of NO and cytokine production, macrophages were incubated in complete culture medium with or without 10 μg/ml LPS. After 24 h, culture supernatants were collected and stored at −70 °C for subsequent NO and cytokine analyses as described below.

Splenocyte isolation and activation
Splenocytes were isolated aseptically as previously described [36] in Krebs–Ringer/Hepes buffer (pH 7.4) supplemented with BSA (5 g/l). Isolated splenocytes (3.0 × 10^6 cells/l) in splenocyte complete culture medium [RPMI 1640 supplemented with 4% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic/antibiotic solution, glutamine (4 mmol/l), Hepes (25 mmol/l) and 2-mercaptoethanol (2.5 μmol/l)] were incubated in sterile 24-well plates for 48 h in a humidified atmosphere at 37 °C in the presence of 5% CO_2. The cell culture medium either contained no mitogen (unstimulated cells) or was supplemented with Con A (5 mg/l) or with PMA (30 μg/l) plus ionomycin (0.75 μmol/l). After 48 h, splenocyte culture supernatants were collected and stored at −70 °C for subsequent NO and cytokine analyses. Unstimulated and stimulated splenocytes were washed twice in Krebs–Ringer/Hepes buffer (pH 7.4) supplemented with BSA (5 g/l), in preparation for indirect single- and double-label immunofluorescence analyses (described below).

Indirect immunofluorescence (phenotype) assay
Peritoneal macrophages and lymphocyte subsets from freshly isolated splenocytes and cultured splenocytes (unstimulated and Con A-stimulated) were identified by indirect immunofluorescence assay [32]. For splenocytes the following monoclonal antibodies to rat cell-surface antigens were used: OX19 (CD5), which recognizes a glycoprotein present on thymocytes and peripheral T cells; w3/25 (CD4), which reacts with a glycoprotein on T helper cells and peritoneal macrophages; OX8 (CD8a), which recognizes thymocytes, T suppressor/cytotoxic and NK cells; OX12, which reacts with a determinant on the rat κ chain of immunoglobulin on B cells; OX42 (CD11b/c), which reacts with a receptor found on most macrophages, monocytes, granulocytes and dendritic cells; 3.2.3, which recognizes the antigen rNKR-P1A on rat NK cells and a subset of T cells; OX26 (CD71), which

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recognizes the transferrin receptor on activated T and B cells and macrophages; OX39 (CD25), which recognizes the α-chain of the IL-2 receptor on activated T and B cells and macrophages; and J319 (CD28), which reacts with a co-stimulatory receptor molecule on T cells. For peritoneal cells, the OX19 and OX42 antibodies were used. Since the monoclonal antibodies were not prelabelled with a fluorescent marker, the cells were subsequently incubated with either fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated goat anti-(mouse IgG). Cells were fixed in paraformaldehyde and the percentage of cells expressing each marker was determined by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA, U.S.A.). The resulting percentages were corrected for background fluorescence using the analysis of cells incubated with fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated goat anti-(mouse IgG) alone; values for background fluorescence were 5 and 0% respectively (results not shown).

**Macrophage cytostatic activity**

Macrophage-sensitive P815 mastocytoma cells were maintained in Dulbecco’s minimal essential medium at 37 °C and 5% CO₂, and the medium was replaced every 2 days during the experiment. Before being used, P815 cells were washed by centrifugation at 380 g for 10 min at 4 °C, counted with a haemocytometer and resuspended in the appropriate volume of macrophage complete culture medium to obtain a density of 0.2 × 10⁶ cells/l.

Macrophage cytostatic activity was measured by a slightly modified version of the method of Lopez et al. [37], based on the inhibition of [methyl-³H]thymidine incorporation by macrophage-sensitive P815 target cells in co-culture with macrophages. P815 cells (4 × 10⁶) were added to various amounts of macrophages to achieve effector/target cell ratios of between 0:1 and 10:1. Immediately after adding the P815 cells, 0.5 μCi of [methyl-³H]thymidine was added to each well. Preliminary experiments showed that a 6 h incubation of the co-culture at 37 °C and 5% CO₂ was sufficient to demonstrate inhibition of thymidine incorporation by P815 target cells (results not shown). After 6 h, cells were harvested on to glass-fibre filters using a multi-well harvester (Skatron Instruments, Lier, Norway) to eliminate non-incorporated radioactivity. The dried filters were dissolved in 4 ml of Ecolite® scintillation fluid, and the radioactivity incorporated into the P815 cells was measured using a Beckman β-radiation counter (LS 5801; Beckman Instruments Inc., Mississauga, ON, Canada).

Macrophage cytostatic activity was determined by taking the value obtained on incubating the target cells alone (i.e. 0:1 ratio) as 100% and applying the following formula:

\[
\text{Macrophage cytostatic activity} = 100 - \left(100 \times \frac{\text{c.p.m. TM}}{\text{c.p.m. T}}\right)
\]

where c.p.m. TM is the radioactivity in the target cell/macrophage co-culture and c.p.m. T is the radioactivity in the target cell culture.

**NK cell cytotoxicity assay**

A 4 h sodium chromate ⁵¹Cr release assay was performed using NK-cell-sensitive YAC-1 cells as targets and freshly isolated splenocytes as effector cells, as previously described [32]. The percentage lysis of the target cells was calculated as:

\[
\text{Specific lysis (％)} = 100 \times \frac{\left(\text{mean experimental } \frac{\text{⁵¹Cr release (c.p.m.)}}{\text{mean spontaneous } \frac{\text{⁵¹Cr release (c.p.m.)}}{\text{mean maximum } \frac{\text{⁵¹Cr release (c.p.m.)}}{\text{mean spontaneous } \frac{\text{⁵¹Cr release (c.p.m.)}}}\right)}}{\text{specific binding. Recombinant IL-2 standards (15–2000 pg/ml) and appropriately diluted splenocyte culture supernatants were then added in triplicate at 100 μl per well, incubated for 4 h at room temperature with PBS/10% (v/v) fetal calf serum to prevent non-specific binding. Recombinant IL-2 standards (15–2000 pg/ml) and appropriately diluted splenocyte culture supernatants were then added in triplicate at 100 μl per well, incubated for 4 h at room temperature, washed, and further incubated with appropriately diluted biotinylated mouse anti-(rat IL-2) for 1 h. After extensive washing, the plates were incubated for 30 min with horseradish peroxidase-conjugated avidin D. The absorbance was measured at 405 nm in a microplate reader (Bio-Tek Instruments, Burlington, VT, U.S.A.) and the concentration of IL-2 in the culture supernatants was quantified by comparison with the standard curve generated with recombinant IL-2.

**Splenocyte IFN-γ and TNF-α production**

IFN-γ and TNF-α concentrations in supernatants collected from unstimulated and stimulated splenocytes were determined using ELISA kits (Genzyme Diagnostics, Cambridge, MA, U.S.A.).

**NO production**

NO production was estimated by analysing nitrite (NO₂⁻), a product of the l-arginine-dependent NO
pathway) concentration in culture supernatants using a colorimetric assay based on the Griess reaction [38]. Briefly, 100 µl of splenocyte or macrophage culture supernatant was combined with 100 µl of Griess reagent (1 vol. of 0.5% sulphanilamide in 6% phosphoric acid plus 1 vol. of 0.05% naphthylethylene-diamine dihydrochloride in distilled water) and incubated for 10 min at room temperature. The absorbance was measured at 540 nm in a microplate reader, and NO\textsuperscript{−} concentrations were determined with reference to a standard curve generated with sodium nitrite (10–100 nmol/ml) in complete culture medium. In some experiments, inhibitors of NO synthase activity, i.e. \(N^G\)-nitro-L-arginine methyl ester (\(L\)-NAME; 120 nmol/10\(^6\) cells) or \(S\)-methylisothiourea (SMT; 120 nmol/10\(^6\) cells), were added to the NK cell cytotoxicity and NO assays.

### Statistical analysis

Results are presented as means ± S.E.M. All statistical analyses were conducted using the SAS statistical package (version 6.11; SAS Institute, Cary, NC, U.S.A.). For phenotype, cytotoxicity, nitric oxide, cytokine, and plasma and tissue amino acid data, the effects of the tumour in rats fed the control amino acid diet were determined by one-way analysis of variance (ANOVA). Similarly, for tumour-bearing rats, the effects of diet were analysed by one-way ANOVA. The method of least-squares means was used to identify significant (\(P < 0.05\)) differences among treatment groups. Food intake, cytostatic activity and cytotoxicity data were also compared among groups by a one-way split-plot (repeated measures) ANOVA [39]. Paired \(t\) tests were used to compare cytokine and NO\textsuperscript{−} production by immune cells in the presence or absence of mitogen.

### RESULTS

#### Food intake and body, tumour and spleen weights

There were no differences between treatment groups in food intake, as shown in Figure 1. However, the food intake of both healthy and tumour-bearing rats started to decline at day 12 (\(P < 0.05\), by repeated-measures ANOVA). Animal body, tumour and spleen weights are presented in Table 2. Initial and final body weight and tumour weight were not significantly different among groups. Spleen weight and the number of spleen cells isolated per g of spleen did not differ between healthy and tumour-bearing rats fed the control diet. For tumour-bearing rats, diet did not significantly affect the number of spleen cells isolated per g of spleen.

#### Biochemical and immunological analysis

Plasma and skeletal muscle amino acid concentrations

There were no significant effects of the tumour or diet on plasma amino acid concentrations (results not shown). However, tibialis anterior muscles isolated from tumour-bearing animals fed the control diet showed significant reductions in free glutamate (−54%), Gln (−30%) and Arg (−25%) compared with those from healthy control rats (Table 3). The largest effect of the diet treatments was on these amino acids in muscles of tumour-bearing rats, as shown in Table 3. Dietary supplementation with Gln or OKG increased muscle Gln concentrations (≥52% and ≥56% respectively; \(P < 0.05\) compared with tumour-bearing control), while Arg elicited a similar, but non-significant, effect. There was not, however, a complete restoration of muscle Gln concentration by any diet treatment. Supplemental Arg and OKG treatments raised muscle Arg concentrations to levels seen in healthy animals, while dietary Gln did not have this effect.

Immune cell phenotypes

Immune cell phenotypes in freshly isolated splenocytes and in splenocytes stimulated with Con A for 48 h are presented in Tables 4 and 5 respectively. The tumour did not significantly affect the proportions of immune cell phenotypes in freshly isolated splenocytes, with the
Table 2  Body, tumour and spleen weights
Buffalo rats were fed semi-purified diets supplemented with Gln, Arg, OKG or a control mixture of non-essential amino acids for 14 days after implantation of the Morris hepatoma 7777. Healthy (control) rats were not implanted with a tumour and were fed a control mixture of non-essential amino acids in the diet. Final body weight includes tumour weight. Values are means ± S.E.M. (n = 7 per group). *Significantly different from tumour-bearing rats fed the control diet (P < 0.04; ANOVA and least-squares means).

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>Diet</th>
<th>Healthy</th>
<th>Tumour</th>
<th>Gln</th>
<th>Arg</th>
<th>OKG</th>
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<tr>
<td>Initial body weight (g)</td>
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<td>190 ± 10</td>
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<td>Spleen weight (mg/100 g body weight)</td>
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<td>216 ± 8</td>
<td>241 ± 8*</td>
<td>228 ± 5</td>
<td>225 ± 7</td>
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<tr>
<td>10^9 × Spleen cells/g of spleen</td>
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<td>380 ± 24</td>
<td>409 ± 23</td>
<td>369 ± 12</td>
<td>378 ± 31</td>
<td>351 ± 18</td>
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</tbody>
</table>

Table 3  Free amino acid concentrations of tibialis anterior muscle
Buffalo rats were fed semi-purified diets supplemented with Gln, Arg, OKG or a control mixture of non-essential amino acids for 14 days after implantation of the Morris hepatoma 7777. Healthy (control) rats were not implanted with a tumour and were fed a control mixture of non-essential amino acids in the diet. Values are means ± S.E.M. (n = 7 per group). Means within a row that do not share a common superscript letter are significantly different (P < 0.05; ANOVA).

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>Diet</th>
<th>Healthy</th>
<th>Tumour</th>
<th>Gln</th>
<th>Arg</th>
<th>OKG</th>
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<td>Glutamate</td>
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<td>2.38 ± 0.31a</td>
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<td>1.30 ± 0.13c</td>
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<td>1.98 ± 0.34b</td>
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<td>2.89 ± 0.33bc</td>
<td>3.10 ± 0.36c</td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>2.28 ± 0.22a</td>
<td>1.72 ± 0.08b</td>
<td>1.56 ± 0.11b</td>
<td>2.37 ± 0.20a</td>
<td>2.05 ± 0.10b</td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td>0.17 ± 0.02a</td>
<td>0.14 ± 0.01a</td>
<td>0.13 ± 0.01a</td>
<td>0.14 ± 0.02a</td>
<td>0.12 ± 0.01a</td>
</tr>
</tbody>
</table>

Table 4  Immune cell phenotypes in freshly isolated splenocytes
Splenocytes were isolated from healthy (control) or tumour-bearing rats fed semi-purified diets supplemented with Gln, Arg, OKG or a control mixture of non-essential amino acids, as indicated. Immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry. Values are means ± S.E.M. (n = 6 per group). *Significant effect of the tumour in rats fed the control diet (P < 0.05; ANOVA). Means within a row that do not share a common superscript letter are significantly different (P < 0.02; ANOVA and least-squares means).

<table>
<thead>
<tr>
<th>Immune cell phenotype</th>
<th>Animal treatment</th>
<th>Diet</th>
<th>Healthy</th>
<th>Tumour</th>
<th>Gln</th>
<th>Arg</th>
<th>OKG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5+ T cells</td>
<td></td>
<td>40 ± 1</td>
<td>61 ± 1</td>
<td>59 ± 1</td>
<td>60 ± 1</td>
<td>60 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD5+ CD4+ T helper cells</td>
<td></td>
<td>32 ± 1</td>
<td>36 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD5+ CD8+ T suppressor/cytotoxic cells</td>
<td></td>
<td>12 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>13 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td></td>
<td>3.3 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>2.8 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td>23 ± 1</td>
<td>24 ± 1</td>
<td>25 ± 1</td>
<td>23 ± 1</td>
<td>24 ± 1</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td>7.7 ± 0.5</td>
<td>6.1 ± 0.2*</td>
<td>7.5 ± 0.4*</td>
<td>7.1 ± 0.5*</td>
<td>7.3 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>NK cells</td>
<td></td>
<td>7.3 ± 0.5</td>
<td>6.0 ± 0.7</td>
<td>6.5 ± 0.4</td>
<td>6.4 ± 0.6</td>
<td>6.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>CD5+ CD28+</td>
<td></td>
<td>59 ± 1</td>
<td>57 ± 1</td>
<td>56 ± 2</td>
<td>56 ± 1</td>
<td>58 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD4+ CD28+</td>
<td></td>
<td>32 ± 1</td>
<td>36 ± 1</td>
<td>35 ± 1</td>
<td>36 ± 2</td>
<td>33 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD8+ CD28+</td>
<td></td>
<td>15 ± 1</td>
<td>16 ± 1</td>
<td>15 ± 2</td>
<td>17 ± 1</td>
<td>17 ± 1</td>
<td></td>
</tr>
</tbody>
</table>
The relative proportion of freshly isolated splenocytes from tumour-bearing rats expressed CD28 compared with healthy rats. The relative proportion of freshly isolated splenocytes expressing the IL-2 receptor (CD25) was negligible (< 1%) and was not significantly different between control and tumour-bearing rats fed the control diet (results not shown). Similarly, the relative proportion of immune cell phenotypes expressing CD25 after Con A stimulation was not significantly affected by the presence of the tumour.

For tumour-bearing rats, diet did not significantly affect the relative proportions of CD25+ cells identified as CD4+ T helper cells, CD8+ T suppressor/cytotoxic cells, B cells, macrophages or CD28+ cells after Con A stimulation. However, after Con A stimulation, tumour-bearing rats fed the OKG-supplemented diet had a higher (P < 0.01) proportion of CD25+ cells compared with rats fed the control or Arg-supplemented diets, but not the Gln-supplemented diet.

### Macrophage cytostatic activity

The purity of the peritoneal macrophage preparations obtained by sterile peritoneal lavage was assessed using the OX19 and OX42 monoclonal antibodies. The proportion of T cells in the peritoneal cell preparations was 81 ± 4% (n = 16), whereas the proportion of macrophages (assessed by OX42) was 34 ± 1% (n = 16). Neither tumour nor diet significantly affected the percentage of macrophages

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**Table 5** Relative proportions of immune phenotypes expressing the IL-2 receptor (CD25) after splenocyte stimulation with Con A for 48 h

<table>
<thead>
<tr>
<th>Immune cell phenotype</th>
<th>Animal treatment…</th>
<th>Diet…</th>
<th>Healthy</th>
<th>Tumour</th>
<th>Gln</th>
<th>Arg</th>
<th>OKG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25+ CD5+ T cells</td>
<td>Control</td>
<td>Control</td>
<td>48±5</td>
<td>51±3*</td>
<td>53±2;*</td>
<td>50±2</td>
<td>56±2</td>
</tr>
<tr>
<td>CD25+ CD4+ T helper cells</td>
<td>Control</td>
<td>Control</td>
<td>37±3</td>
<td>37±3</td>
<td>40±2</td>
<td>36±2</td>
<td>40±2</td>
</tr>
<tr>
<td>CD25+ CD8+ T suppressor/ cytotoxic cells</td>
<td>Control</td>
<td>Control</td>
<td>15±2</td>
<td>18±1</td>
<td>17±1</td>
<td>18±1</td>
<td>19±1</td>
</tr>
<tr>
<td>CD25+ B cells</td>
<td>Control</td>
<td>Control</td>
<td>32±5</td>
<td>32±3</td>
<td>34±4</td>
<td>31±4</td>
<td>30±3</td>
</tr>
<tr>
<td>CD25+ macrophages</td>
<td>Control</td>
<td>Control</td>
<td>6.8±0.9</td>
<td>7.4±1.1</td>
<td>7.9±1.2</td>
<td>10.5±1.7</td>
<td>9.3±1.4</td>
</tr>
<tr>
<td>CD25+ CD28+ cells</td>
<td>Control</td>
<td>Control</td>
<td>45±4</td>
<td>48±4</td>
<td>52±3</td>
<td>50±2</td>
<td>49±2</td>
</tr>
</tbody>
</table>

---

**Figure 2** Macrophage cytostatic activity against P815 cells at different effector/target cell ratios

Peritoneal macrophages were co-cultured with P815 target cells in the presence of [methyl-3H]thymidine. After 6 h cell harvests, and the radioactivity incorporated into the target cells was determined. Macrophage cytostatic activity was calculated as described in the Materials and methods section. Results are means ± S.E.M. (n = 7 per group). A significant effect of the tumour in rats fed the control amino acid diet was determined by a one-way split-plot (repeated measures) ANOVA (+ P < 0.05). Similarly, a significant effect of diet in tumour-bearing rats was determined by a one-way split-plot (repeated measures) ANOVA (* P < 0.05).
found in cells obtained by peritoneal lavage. Macrophage cytostatic activity against P815 cells was lower (30%; \( P < 0.05 \)) for tumour-bearing rats fed the control diet compared with healthy control rats, as determined by a one-way split-plot (repeated measures) ANOVA (Figure 2). A significant effect of diet was also determined by a one-way split-plot (repeated measures) ANOVA; tumour-bearing rats fed the OKG-supplemented diet had higher (39%; \( P < 0.05 \)) macrophage cytostatic activity compared with tumour-bearing rats fed the control diet, but not those fed the Gln- or Arg-supplemented diets (Figure 2).

**Mechanisms of immune cell cytotoxicity and cytostasis**

**Splenocyte cytokine production**

The production of IL-2, IFN-\( \gamma \) and TNF-\( \alpha \) was higher from splenocytes stimulated with Con A or PMA + ionomycin for 48 h compared with unstimulated cells, regardless of treatment or diet (\( P < 0.05 \), by paired \( t \) test; results not shown). Neither tumour nor diet significantly affected the amounts of IL-2, IFN-\( \gamma \) and TNF-\( \alpha \) produced by mitogen-stimulated splenocytes. The overall means for the amounts of IL-2, IFN-\( \gamma \) and TNF-\( \alpha \) produced (\( n \geq 35 \) per cytokine) were 4595 ± 159, 3671 ± 242 and 558 ± 26 pg/ml respectively (after Con A stimulation) and 12538 ± 661, 2693 ± 189 and 706 ± 36 pg/ml respectively (after PMA + ionomycin stimulation).

**NO production**

(a) Splenocytes. Neither tumour nor diet significantly affected the amount of \( \text{NO}_2^- \) produced by unstimulated splenocytes (results not shown). \( \text{NO}_2^- \) production by mitogen-stimulated splenocytes is presented in Figure 4.
Figure 5 Effects of NO synthase inhibitors on NK cell cytotoxic activity in tumour-bearing rats

Splenocytes were incubated for 10 min at 37 °C without NO synthase inhibitors (maximal response) or with either L-NAME (1200 nmol/10^6 cells) or SMT (120 nmol/10^6 cells). Splenocytes were then cultured with ^1^C-labelled YAC-1 cells for 4 h to achieve effector/target cell ratios of between 25:1 and 100:1. NK cell cytotoxic activity is expressed as a percentage of maximal response at the 100:1 ratio. Results are means ± S.E.M. For each diet group, the effect of the NO synthase inhibitor on NK cell cytotoxic activity was determined by one-way ANOVA (*P < 0.05 compared with maximal response).

The presence of a tumour did not significantly affect NO_2^- production by stimulated splenocytes when rats were fed the control diet. Following Con A stimulation, splenocytes from tumour-bearing rats fed the OKG-supplemented diet produced higher concentrations of NO_2^- (P < 0.02) compared with splenocytes from tumour-bearing rats fed either the control diet or the Arg-supplemented diet. Following stimulation by PMA + ionomycin, splenocytes from tumour-bearing rats fed the OKG-supplemented diet produced higher amounts of NO_2^- (+28%; P < 0.04) compared with tumour-bearing rats fed the control diet.

L-NAME and SMT inhibited splenocyte NO_2^- production for up to 48 h (results not shown). L-NAME inhibited (P < 0.05) NK cell cytotoxicity consistently in splenocytes isolated from tumour-bearing rats fed the control, Arg- or OKG-supplemented diets (Figure 5). This assay was not performed on splenocytes from the Gln-supplemented group. SMT did not inhibit NK cell cytotoxicity in any of the dietary groups (Figure 5).

(b) Macrophages. In all groups, except the Gln-supplemented diet group, NO_2^- production was higher from macrophages stimulated with LPS compared with that from unstimulated cells (P < 0.05, by paired t test). The tumour did not significantly affect the amount of NO_2^- produced by unstimulated or LPS-stimulated macrophages when rats were fed the control diet (Figure 6). Both unstimulated and LPS-stimulated macrophages from tumour-bearing rats fed the OKG-supplemented diet produced higher concentrations of NO_2^- (P < 0.05) than macrophages from tumour-bearing rats fed the Arg-supplemented diet (Figure 6).

**DISCUSSION**

**Nutritional and immune status of tumour-bearing animals**

In previous studies we have examined animals during progressive growth of the Morris hepatoma 7777 [7,28,35]. At 2 weeks post-implantation, tumours attain ~0.8% of body mass, and food intake and nitrogen balance begin to decline. During the third week, when there is a marked deterioration in food intake and nitrogen balance, changes in body weight are almost entirely attributable to a rapid increase in tumour mass. Together, the rate of protein deposition and amino acid oxidation by the tumour may account for in excess of 70% of daily protein intake by the host during the third week after implantation [28]. These results suggest that the nutritional status of animals bearing tumours can change from moderate to poor to catastrophic in a short period of time. Thus host nutritional status must be carefully defined under the experimental conditions used, so as to clearly distinguish the effects of altered nutritional status on measured variables. Dietary supplementation with...
Gln, Arg and OKG has been studied for a wide range of tumour types and burdens (e.g. [10,11,15,20–22,27,28, 30,40–42]). Results from these studies are nearly impossible to compare, due to vast differences in nutritional status of the animals studied. We selected a defined, limited stage of cancer associated with lowered amino acid levels in skeletal muscle pools [35] for comparison of the effects of Gln, Arg and OKG supplementation.

Of the immune indices measured, the major changes elicited by the tumour-bearing state were evident in macrophages. Specifically, both the proportion of macrophages in the spleen and the macrophage cytostatic activity were reduced at 2 weeks after tumour implantation; the combination of these effects is a substantial reduction in overall macrophage anti-tumour defence. Suppression of macrophage cytostatic activity during progressive tumour growth has been demonstrated in other models [5]. One potential mechanism may be a reduced capacity of macrophages from tumour-bearing hosts to produce NO [5], a finding that has been associated with diminished expression of inducible NO synthase [43]. In the present study, production of NO by peritoneal macrophages was not affected by the tumour. However, it cannot be concluded from this study whether other mechanisms, such as the production of NO by tumour-infiltrating macrophages or the production of prostaglandin E\(_2\) [40], are involved in suppression of macrophage activity in this tumour model.

**Diet and immune function**

Recent investigation has focused on supplementing enteral or total parenteral nutrition with various immune-enhancing nutrients, including amino acids [7,25], either alone or as part of multiple nutrient formulations [44]. Unfortunately, the design of most studies has been such that it is not possible to draw conclusions as to the efficacy of individual nutrients for improving immunity. The present study is the first to compare the relative efficacy of Gln, Arg and OKG for improving immune defence in tumour-bearing rats. Overall, the effects of diet on the immune indices measured were limited. In particular, Gln and Arg had minimal effects on spleen cell phenotype distribution, and did not induce significant alterations in macrophage or NK cell cytotoxicity, or in NO or cytokine production. By contrast, OKG appeared to enhance certain immune functions, albeit to a limited extent. Tumour-bearing rats fed the OKG-supplemented diet had increased (+22%) NK cell cytotoxicity, but showed no change in the proportion of NK cells in the spleen, suggesting that OKG enhanced the function of NK cells. Tumour-bearing rats fed the OKG-supplemented diet had higher (+39%) macrophage cytostatic activity, indicating a general enhancing effect of OKG on cell-mediated cytotoxic capacity. In effect, feeding tumour-bearing rats with OKG counteracted the reduction in macrophage cytostatic activity seen at 2 weeks after tumour implantation. Our results are in agreement with those of Albina [26], who showed that feeding healthy rats on OKG (6.15 g·kg\(^{-1}\)·day\(^{-1}\)) increased macrophage cytotoxicity *in vitro*.

There are multiple putative effectors of macrophage and NK cell cytotoxicity. We attempted to explain observed diet-induced differences in cytotoxicity in terms of immune cell production of cytokines and NO, compounds that may be factors in the up-regulation of immune cell cytotoxicity [2,45]. Tumour-bearing rats fed the OKG-supplemented diet had a higher (+10%) proportion of total T cells expressing the IL-2 receptor (CD25\(^{+}\)) after mitogen stimulation. However, diet did not significantly affect splenocyte IL-2, IFN-\(\gamma\) or TNF-\(\alpha\) production, suggesting that diet-induced differences in cytotoxicity are not mediated by cytokines. In the OKG-supplemented group, the enhanced killing ability of NK cells was associated with higher splenocyte NO production. To investigate NO as a potential mediator of NK cell cytotoxicity, we used two inhibitors of NO synthase, SMT, a specific inhibitor of inducible NO synthase, did not inhibit NK cell cytotoxicity, even though it was effective in suppressing splenocyte nitrite production. Incubations with L-NAME, a non-specific NO synthase inhibitor, resulted in a reproducible inhibition (~15%) of cytotoxicity, which is consistent with results in the literature [46]. However, the fact that SMT is ineffective in inhibiting NK cell cytotoxicity, along with suggestions that many of the NO synthase inhibitors (including L-NAME) may exert non-specific effects through interactions with other iron-containing enzymes [47], implies that NK cell cytotoxicity is not mediated by NO. Thus we are presently lacking a complete description of immune cell cytotoxicity mechanisms and the role of amino acids therein. While this awaits further study, the possible role of OKG in the generation of polyamines [48], as well as its role in reducing hyperammonaemia [49], should be investigated as alternative mechanisms in relation to anti-cancer immune defence.

**Diet and tumour growth**

Feeding rats on a nutritionally complete, semi-purified diet supplemented with Gln, Arg or OKG had no net effect on growth of the Morris hepatoma 7777 compared with a control mixture of non-essential amino acids. Animals bearing a tumour showed specific depletion of intracellular Gln, glutamate and Arg pools in skeletal muscle, and these were temporarily associated with reductions in macrophage numbers and cytostatic activity. Diet treatments were associated with positive changes in muscle free Gln, glutamate and Arg pools, but only OKG was associated with a significant improvement in immune variables in tumour-bearing animals. Overall,
the results suggest no net advantage for anti-tumour immunity of these diet treatments in this model.

It has been proposed that when tumour cells are highly susceptible to recognition and destruction by the host immune system, the ability of Arg to up-regulate anti-tumour defence can result in a decrease in tumour growth [29]. Since the Morris hepatoma is modestly immunogenic [34], a logical next step would be to compare the effects of dietary Gln, Arg and OKG on tumours expressing a greater degree of immunogenicity. Such a systematic approach would begin to lend understanding to a literature which is presently very difficult to interpret. There are currently at least 30 published papers focusing on Gln, Arg and OKG supplementation and tumour growth. In the Gln literature, some studies have found no effect of Gln on tumour growth (e.g. [14,15,30,41]), while others have shown tumour growth inhibition by supplemental Gln (e.g. [10,11,40]). Arg supplementation has been variously reported to have no effect on tumour growth (e.g. [42]), to stimulate it (e.g. [20–22]) or to inhibit it (e.g. [17,18,29]). In two reports to date, dietary OKG has not affected tumour growth [27,28]. Individual studies have used a wide variety of tumour types, hosts and tumour immunogenicity, both enteral and parenteral feeding regimens, levels of amino acid supplementation ranging from 1 to 10% (w/w) of the diet, tumour burdens ranging from <1% to 30% of host body weight, and a variety of ‘control diets’. Given this diversity, there is in most cases no obvious explanation as to why specific amino acids might increase or decrease tumour growth in different experimental situations. At present we have no explanation as to why Gln inhibited tumour growth in a previous experiment in our laboratory [10]. It may be that a number of variables, for which we currently cannot account, impact upon the balance between stimulation and inhibition of tumour growth by dietary amino acid supplementation.

Further understanding of the roles of individual amino acids in specific functions of immune and tumour cells would provide clarification of the immunomodulatory potential of amino acids in cancer. The roles of amino acids may be complex. For example, the NO-producing breast cancer cell line (EMT-6) studied by Edwards et al. [21] is potently stimulated by Arg supplementation. If anti-tumour defences are also susceptible to Arg supply, through either its role as a NO precursor or other mechanisms, then it is clearly the ‘balance of power’ between these pro- and anti-tumour effects which becomes important. In the absence of differences in tumour growth, the potential importance of diet-associated changes in the immune variables measured is not clear. However, the ability of specific nutrients to enhance the immune system should not be overlooked, since improved host immunity may be beneficial in terms of prevention of disease recurrence, metastasis or infectious complications, and may be important in host tolerance to immunosuppressive treatments such as chemotherapy. Our results do not preclude such benefits; however, these must be tested in future studies.

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