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Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy.

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Research Article

An interferon-gamma, tumor necrosis factor, and interleukin-1-inducible, high-output pathway synthesizing nitric oxide (NO) from L-arginine was recently identified in rodents. High-dose interleukin-2 (IL-2) therapy is known to induce the same cytokines in patients with advanced cancer. Therefore, we examined renal cell carcinoma (RCC; n = 5) and malignant melanoma (MM; n = 7) patients for evidence of cytokine-inducible NO synthesis. Activity of this pathway was evaluated by measuring serum and urine nitrate (the stable degradation product of NO) during IL-2 therapy. IL-2 administration caused a striking increase in NO generation as reflected by serum nitrate levels (10- and 8-fold increase [P less than 0.001, P less than 0.003] for RCC and MM patients, respectively) and 24-h urinary nitrate excretion (6.5- and 9-fold increase [both P less than 0.001] for RCC and MM patients, respectively). IL-2-induced renal dysfunction made only a minor contribution to increased serum nitrate levels. Metabolic tracer studies using L-[guanidino-15N₂]arginine demonstrated that the increased nitrate production was derived from a terminal guanidino nitrogen atom of L-arginine. Our results showing increased endogenous nitrate synthesis in patients receiving IL-2 demonstrate for the first time that a cytokine-inducible, high-output L-arginine/NO pathway [...]

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Evidence for Cytokine-inducible Nitric Oxide Synthesis from L-Arginine in Patients Receiving Interleukin-2 Therapy

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Abstract

An interferon- γ , tumor necrosis factor, and interleukin-1-inducible, high-output pathway synthesizing nitric oxide (NO) from L-arginine was recently identified in rodents. High-dose interleukin-2 (IL-2) therapy is known to induce the same cytokines in patients with advanced cancer. Therefore, we examined renal cell carcinoma (RCC; $n = 5$) and malignant melanoma (MM; $n = 7$) patients for evidence of cytokine-inducible NO synthesis. Activity of this pathway was evaluated by measuring serum and urine nitrate (the stable degradation product of NO) during IL-2 therapy. IL-2 administration caused a striking increase in NO generation as reflected by serum nitrate levels (10- and 8-fold increase [$P < 0.001$, $P < 0.003$] for RCC and MM patients, respectively) and 24-h urinary nitrate excretion (6.5- and 9-fold increase [both $P < 0.001$] for RCC and MM patients, respectively). IL-2-induced renal dysfunction made only a minor contribution to increased serum nitrate levels. Metabolic tracer studies using L-[guanidino- ^{15}N]₂arginine demonstrated that the increased nitrate production was derived from a terminal guanidino nitrogen atom of L-arginine. Our results showing increased endogenous nitrate synthesis in patients receiving IL-2 demonstrate for the first time that a cytokine-inducible, high-output L-arginine/NO pathway exists in humans. (*J. Clin. Invest.* 1992; 89:867-877.) Key words: acute renal failure • malignant melanoma • nitrate • nitrite • renal cell carcinoma

Introduction

Many host responses to microbial agents and neoplastic cells are mediated by cytokines. Interleukin 2 (IL-2), which is primarily synthesized by activated T lymphocytes, has a central role in the development of cell-mediated immunity (1) and is a key factor in the induction of a complex network of cytokines that include tumor necrosis factor/cachetin (TNF),¹ inter-

feron- γ (IFN γ), interleukin 1 (IL-1), and interleukin 6 (2-7). It was observed, in both mice and humans, that normal resting lymphocytes treated with high concentrations of IL-2 developed the ability to kill a wide range of syngeneic and allogeneic tumor cells (8). IL-2-activated cells were functionally defined as lymphokine-activated killer (LAK) cells and administration of IL-2 plus LAK cells to mice caused regression of established syngeneic tumors (9). Clinical trials of IL-2 plus LAK cells or of IL-2 alone demonstrated objective regressions in ~ 25-33% of patients including 5-10% complete remissions in patients with advanced melanoma or renal cell carcinoma (10). Clinical use of IL-2 therapy, with or without LAK cells, has been limited by the low rate of complete remissions and by severe toxicity that requires intensive care monitoring. Patients receiving IL-2 therapy often develop potentially life-threatening side effects that include fever, chills, hypotension, capillary leak syndrome, as well as evidence of multiple organ system dysfunction, specifically including acute renal insufficiency and cholestatic jaundice (10).

Studies of rodent cytotoxic activated macrophages as an effector component of cell-mediated immunity resulted in the identification of a cytokine (IFN γ and TNF)-inducible pathway that was capable of producing high levels of nitric oxide (NO) and L-citrulline synthesis from L-arginine (11-18) (for reviews, see references 19 and 20). NO is a short-lived paramagnetic lipid-soluble gas which, when present in high concentration in the cellular environment, causes widespread iron-sulfur nitrosyl complex formation that inhibits certain iron-dependent enzymes (21, 22) (Fig. 1). NO has been shown to be a biochemical effector of L-arginine-dependent cell-mediated immune responses to neoplastic cells (11-13, 16, 17, 19) as well as to certain helminths and intracellular pathogens (20, 23-29) in rodent cells in vitro.

A constitutive low-output pathway synthesizing small amounts of NO and L-citrulline from L-arginine was also recently identified (30-33). NO synthesized from L-arginine by the constitutive NO synthase functions as an intercellular and/or intracellular signal. For example, NO synthesized by the constitutive isoenzyme of endothelial cells targets a heme moiety associated with soluble guanylate cyclase of vascular smooth muscle cells and mediates functional linkage between these two cell types (34). Examples of constitutive NO synthase activity include modulation of vascular tone (30) and neurotransmission (31-33).

NO synthesized by either isoenzyme undergoes oxidative degradation to the stable inorganic nitrogen oxides nitrite (NO_2^-) and nitrate (NO_3^-). NO_2^- entering the vascular system reacts rapidly with oxyhemoglobin (35). This results in stoichiometric formation of methemoglobin and NO_3^- from oxyhemoglobin and NO_2^- (see Fig. 1). Therefore, NO synthesized

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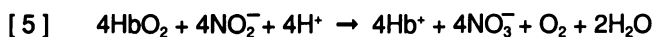
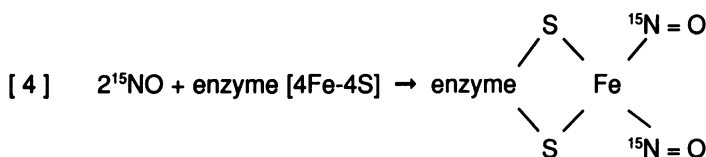
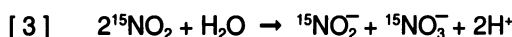
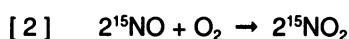
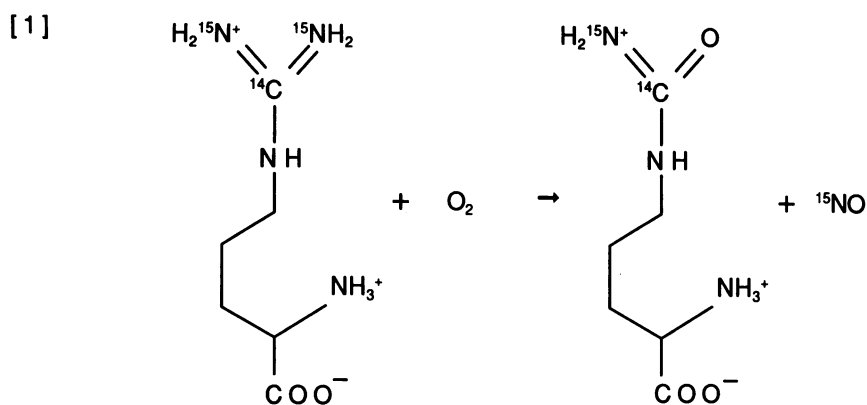
1. Abbreviations used in this paper: BUN, blood urea nitrogen; FE, fractional excretion; GC/MS, gas chromatography-mass spectrometry; LAK, lymphokine-activated killer; MM, malignant melanoma; RCC, renal cell carcinoma; TNF, tumor necrosis factor; TPN, total parenteral nutrition.

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from a terminal guanidino nitrogen atom of L-arginine is detected in serum or urine as NO_3^- . The study reported here was undertaken to examine (a) whether IL-2 administration to patients with cancer caused a rise in NO synthesis from L-arginine; (b) whether urine and serum NO_3^- levels could serve as markers of IL-2-stimulated NO generation; and (c) the extent that IL-2-induced renal dysfunction (36, 37) contributed to elevations in serum NO_3^- levels.

Methods

Subjects. The study protocol was reviewed and approved by the University of Utah School of Medicine Institutional Review Board and written informed consent was obtained from all patients. The patients ($n = 12$) were admitted to the University Hospital's Clinical Research Center for study and treatment (Table I). The patients had metastatic malignant melanoma (MM [$n = 7$; mean age 44 yr, range 24–63]) or metastatic renal cell carcinoma (RCC [$n = 5$; mean age 53 yr, range 39–63]) that was unresponsive to conventional treatment. All the patients with RCC had fully recovered from a unilateral nephrectomy before IL-2 therapy. There were three female MM patients and one female RCC patient enrolled in this study.

High-dose bolus IL-2 treatment with or without LAK cells. Patient eligibility included the following criteria: pathologic confirmation of tumor histology, measurable disease, Karnofsky performance status $> 70\%$, normal stress multiple-uptake gated acquisition or thallium scan, normal pulmonary function (forced expiratory volume in [FEV₁] $> 75\%$ of predicted), normal bone marrow, and liver and renal func-

Figure 1. Precursor and products of the biological synthesis of inorganic nitrogen oxides and L-citrulline from L-arginine. ^{15}N -containing products derived from L- ^{15}N -arginine were identified by GC/MS (13–15, 30) electron paramagnetic resonance spectroscopy (21, 22) except for nitrogen dioxide (NO_2), which was detected by another method (13). The direct synthesis of L-citrulline from L-arginine has been identified with several techniques (12–15, 58). The experiments utilizing L-[guanidino- ^{14}C]arginine (13, 58) are illustrated in the figure. NO formed by reaction [1] can undergo oxidative degradation in aqueous solution (reactions [2] and [3]) or react with nonheme iron associated with sulfur atoms to form nitrosyl-iron-sulfur complexes (reaction [4]). Although not shown, certain other forms of intracellular iron also complex with NO. (Hibbs et al., unpublished data). NO_2^- entering the vascular system reacts rapidly with oxyhemoglobin (35). This results in the stoichiometric formation of methemoglobin and NO_3^- from oxyhemoglobin and NO_2^- (reaction [5]).

tion. The creatinine clearance of MM patients was normal (mean = 122 ml/min). The creatinine clearance of RCC patients with unilateral nephrectomies was $\sim 50\%$ of normal (mean = 59 ml/min). Exclusion criteria included pregnancy, active infection (including HIV), steroid therapy, organ allografts, brain metastases, or prior malignancy. All patients were treated for anticipated IL-2 toxicity with acetaminophen 650 mg p.o. every 4 h, indomethacin 25 mg p.o. every 6 h, and ranitidine 150 mg i.v. every 12 h. Patients also received meperidine 25–50 mg every 4 h i.v. as needed for chills due to IL-2 or LAK cell administration, as well as symptomatic treatment for pruritus, vomiting, or diarrhea as required. Each patient received highly purified recombinant human IL-2 from *Escherichia coli* (sp act 18×10^6 IU/mg) generously provided by Cetus Corp., Emeryville, CA, at 600,000 IU/kg i.v. every 8 h on days 1–5 (induction course). The IL-2 used in these experiments contained < 0.012 ng/ml LPS by *Limulus* ameocyte lysate assay (18×10^6 IU of IL-2 in 1 ml of PBS). Patients received either IL-2 alone, or IL-2 in conjunction with LAK cells during days 11–15 (consolidation course), Table I. All five patients with RCC received induction IL-2 therapy. Patient 1 was not given a consolidation course of IL-2 because of a severe decrease in renal function.

Patients who randomized to IL-2 with LAK cells were leukapheresed for 5 h/d on days 7–10. The cells from each pheresis were cultured for 3–4 d in vitro in serum-free Ex Vivo-10 medium (M. A. Bioproducts, Walkersville, MD) containing 2 mM L-glutamine and 1,500 U of IL-2/ml, and readministered to the patient in a single i.v. daily infusion, (in 250 ml of saline containing 5% albumin and 50,000 U of IL-2) during the second (consolidation) IL-2 treatment course on days 11, 12, and 14. Doses of IL-2 were held for National Cancer Institute common toxicity grade III or IV toxicity, usually oliguria, hypotension

Table I. Characteristics of Patients Treated with IL-2

Patient	Diagnosis*	Age/Sex	Date of treatment	IL-2 doses		Total IU IL-2 $\times 10^6$	Total LAK cells $\times 10^{10}$	Clinical response (duration) [‡]
				Induction	Consolidation			
								mo
1	RCC	63/M	8/8/89	10	0	456	0	PD
2	RCC	51/M	9/5/89	13	7	2856	23.8	PD
3	RCC	60/M	9/26/89	11	8	935	0	CR (>22)
4	RCC	39/F	11/21/89	12	7	627	12.4	PD
5a [§]	MM	24/F	2/20/90	11	7	702	9.2	—
5b	MM		5/29/90	13	6	741	8.8	PR (11)
6	MM	45/M	3/6/90	12	11	1173	14.0	PD
7	RCC	54/M	3/13/90	7	3	444	6.9	SD (7)
8	MM	63/F	4/17/90	12	9	1046	0	PD
9	MM	45/F	6/2/90	11	6	745	0	PR (2)
10	MM	48/M	6/26/90	14	13	1118	18.1	SD (2.5)
11	MM	51/M	7/3/90	13	10	773	10.7	PD
12	MM	35/M	9/19/90	14	5	744	0	PD

* RCC, renal cell carcinoma; and MM, malignant melanoma.

[‡] PD, progressive disease; SD, stable disease; PR, partial response; and CR, complete response.

[§] Patient 5 was administered two complete courses of IL-2.

requiring $> 4 \mu\text{g/kg} \cdot \text{min}$ dopamine, severe hypoxia, or neurologic toxicity. Central venous pressure and systemic blood pressure were measured every 4 h, during hospitalization. Our patients were monitored closely for evidence of infection (urine, blood, and intravascular device cultures for oral temperatures $> 38^\circ\text{C}$). Three episodes of line infection were documented in two patients (coagulase-negative *Staphylococcus* [patient 4] and coagulase-negative *Staphylococcus* followed by *Proteus mirabilis* [patient 6]).

Nutrition regimen. All patients received parenteral nutrition (TPN) infused via a central line. The TPN solution (a mixture of equal parts of 8.5% Aminosyn [Abbott Laboratories, North Chicago, IL] and 50% dextrose plus standard vitamin and mineral solutions, and infused at a rate of $\sim 100 \text{ ml/h}$ [standard TPN regimen]) consistently tested negative for NO_2^- and NO_3^- . Lipids (Liposyn, Abbott Laboratories) were given two times per week. The standard TPN regimen provided 10.2 g of L-arginine daily.

Low $\text{NO}_2^-/\text{NO}_3^-$ foods were also provided in small amounts to those patients desiring oral feedings. Carnation Instant Breakfast (Carnation Co., Los Angeles, CA) (laboratory analysis showed $< 0.3 \text{ mg}$ of NO_3^- per serving), or other low $\text{NO}_2^-/\text{NO}_3^-$ foods were supplied, based on limited information on food $\text{NO}_2^-/\text{NO}_3^-$ content (38, 39). The following food groups were excluded: cured meats, malt, vegetables raw or cooked, including potatoes, melons, fish, imported cheese, and any fermented foods. The diet included milk, ice cream and domestic cheese, eggs, breads and cereals (including whole kernel corn), fresh meats (chicken, turkey, beef and pork), legumes, other fruits, hard candy and carbonated beverages, sugar, coffee, salt, and pepper. Daily enteral intake was also recorded and included in the calorie counts.

Measurement of urine and blood NO_3^- . Sequential 24-h urine specimens were collected throughout the study period. Bacterial growth in the urine during the collection period was prevented by placing 200 ml of 2-propanol into a 3-liter collection bottle. The samples were frozen at -80°C until a 1:10 dilution of urine was assayed for NO_3^- as previously described (24, 40). The 2-propanol did not affect the enzymatic conversion of NO_3^- to NO_2^- by the *E. coli* NO_3^- reductase. Serum was diluted 1:1 with $\text{NO}_2^-/\text{NO}_3^-$ free distilled water and then ultrafiltered (Ultrafree MC microcentrifuge device, UFC3 LGC NB, Millipore Corp., Bedford, MA) at 2,000 g for 120 min. NO_3^- in the filtrate was reduced to NO_2^- by mixing 80 ml with 80 ml *E. coli* NO_3^- reductase

suspension prepared and then incubated for 1 h at 37°C . The *E. coli* NO_3^- reductase had no NO_2^- reductase activity when prepared as described (24). The reaction mixture was centrifuged (12,000 rpm, Microfuge, Beckman Instruments, Inc., Fullerton, CA) for 5 min. Supernatant (50 ml) was added to 100 ml of Griess reagent (24). Absorbance (A540) was measured on a microplate reader (Dynatech Laboratories, Inc., Chantilly, VA).

Administration of the stable nonradioactive isotope L-arginine HCl (guanidino $^{15}\text{N}_2$, 70%). The material supplied by Cambridge Isotope Laboratories, Woburn, MA, contained 70% L-[guanidino- $^{15}\text{N}_2$]-arginine (L-[^{15}N]arginine) as determined by potentiometric titration (Galbraith Laboratories, Knoxville, TN). HPLC and nuclear magnetic resonance analysis showed 1.5% L-ornithine and $< 2\%$ of the optical isomer D-[guanidino $^{15}\text{N}_2$]arginine which is not a substrate for the cytokine-induced L-arginine/NO pathway (11, 12). The remaining and major other component was unlabeled ammonium chloride. A 70 mM stock solution of L-[^{15}N]arginine prepared in sterile pyrogen-free water contained $< 1 \text{ ng/ml}$ of lipopolysaccharide (LPS) as measured by the *Limulus* amoebocyte lysate assay. This stock solution was filtered three times (0.22 μm , Millipore Corp.) and sterility was confirmed by culture on blood agar. L-[^{15}N]arginine HCl was added to a TPN solution containing a 1:1 mixture of 5.4% Nephramine (an essential amino acid mixture that lacks L-arginine; Kendall McGraw Laboratories, Inc., Irvine, CA) and 50% dextrose plus vitamins/electrolytes. The standard TPN mixture was discontinued and the L-[^{15}N]arginine/Nephramine containing TPN mixture (2,400 ml) infused over a 24-h period. The standard TPN regimen was resumed upon completion of the L-[^{15}N]arginine infusion. The patients tolerated the L-[^{15}N]arginine infusions without incident or adverse effects. All calculations of ^{15}N enrichment of serum NO_3^- , urine NO_3^- , and urine urea were based on the administration of a 70% L-[^{15}N]arginine solution.

^{15}N enrichment of serum NO_3^- , urine NO_3^- , and urine urea. Atom percent excess of urine and serum [^{15}N] NO_3^- and [^{15}N]urea was measured by gas chromatography-mass spectrometry (GC/MS) (model 5971, Hewlett-Packard Co., Palo Alto, CA), using electron ionization as previously described (41, 42). GC/MS analysis was performed by Dr. David A. Wagner, Metabolic Solutions Inc., Acton, MA.

Determination of renal function. Glomerular filtration rate was monitored throughout by daily determination of endogenous creati-

nine clearance. Renal handling of NO_3^- , urea, sodium, and water was assessed as previously reported (43, 44).

Statistical analysis. Statistical analysis was conducted using stepwise regression to evaluate differences among patients, followed by isolation of treatment effects as post-patient factors. Stepwise analysis of covariance procedure was used to compare periods of IL-2 administration vs. periods when patients were not receiving the drug. Two levels of prior comparisons were used. The first level tested for the presence of a linear (monotonically increasing or decreasing) trend within the four treatment phases of the study (two IL-2 and two off-drug) (45, 46). In the second level, paired comparisons for differences among treatments were conducted, each averaging across the 5 d of observations within a treatment phase. The first compared the first IL-2 treatment with the first off-drug phase. The second compared the second IL-2 treatment with the second off-drug phase. The third contrast compared the first two treatment phases with the second two treatment phases and constituted a test of the reversibility and reproducibility of IL-2 therapy. Additional analyses were conducted to compare all possible pairs of treatments. Means for the four treatment phases were subjected to analysis of variance for repeated measurements. In case of overall significance, Newman-Keul's pairwise comparison of the differences between treatments was performed. All data are expressed as mean $\pm$ SE. A P value of < 0.05 was taken to indicate significant differences.

Results

Baseline serum NO_3^- and 24-h urine NO_3^- output. Serum NO_3^- levels in six normal control individuals (four males and two females; mean age 38 yr) was 32 ± 4 $\mu\text{mol/liter}$. The control individuals consumed a low nitrite ($\text{NO}_2^-/\text{NO}_3^-$) oral diet for 24 h and then fasted overnight (12 h) before donating blood for the NO_3^- assay. In the current study, pretreatment serums were assayed for NO_3^- . Baseline serum NO_3^- for MM patients was 32 ± 3.3 $\mu\text{mol/liter}$ ($n = 9$) and for RCC patients 36 ± 3.2 $\mu\text{mol/liter}$ ($n = 5$). The mean 24-h urinary NO_3^- output for the patients on TPN before initiation of IL-2 therapy was 600 ± 80 μmol ($n = 5$) for MM patients and 521 ± 71 μmol ($n = 3$) for RCC patients. This was similar to urinary NO_3^- excretion (690 $\mu\text{mol/24 h}$) in eight healthy subjects with unrestricted activity (mean age 21 yr) on a low protein and low $\text{NO}_2^-/\text{NO}_3^-$ oral diet (daily NO_3^- ingestion < 180 μmol) (47).

Effect of IL-2 therapy on serum NO_3^- levels. The 5-d induction course of IL-2 therapy caused a significant rise of serum NO_3^- levels in both RCC patients (Fig. 2, left, panel a) and MM patients (Fig. 2, right, panel a). The results for RCC and MM patients are presented separately because of the possible effects of unilateral nephrectomy on serum and urinary NO_3^- levels. Serum NO_3^- of RCC patients increased from a baseline level of 36 ± 3.2 to 106 ± 16 $\mu\text{mol/liter}$ on day 3 of therapy, followed by a further increase on day 5 (313 ± 55 $\mu\text{mol/liter}$), and peaked on day 7 (353 ± 31 $\mu\text{mol/liter}$). This 10-fold rise in serum NO_3^- was highly significant ($P < 0.001$). The overall pattern of serum NO_3^- changes in MM patients was similar to that observed in RCC patients. Serum NO_3^- levels increased from the baseline of 32 ± 3.3 $\mu\text{mol/liter}$ to 57 ± 35 , 189 ± 48 , and 247 ± 96 on days 4, 5, and 6, respectively ($P < 0.003$). The highest individual serum NO_3^- level recorded was 646 $\mu\text{mol/liter}$ on day 7. Serum NO_3^- decreased to 25 ± 4 $\mu\text{mol/liter}$ by day 11 when consolidation IL-2 therapy was started. During the interval between the induction course and consolidation course of IL-2, serum NO_3^- levels fell more slowly in RCC patients than in MM patients. During the second half of the treatment course peak serum

NO_3^- for MM patients was 122 ± 23 $\mu\text{mol/liter}$ (day 15). The peak serum NO_3^- level during the consolidation course of IL-2 was significantly less than during the induction course both in RCC ($P < 0.02$) and MM ($P < 0.05$) patients.

Effect of IL-2 therapy on endogenous NO_3^- synthesis as determined by 24-h urinary NO_3^- excretion. Daily NO_3^- excretion paralleled serum NO_3^- levels. Fig. 2, both panels b, show the mean 24-h urinary NO_3^- excretion for patients with RCC and MM, respectively. 24-h NO_3^- excretion rose significantly ($P < 0.001$) from a baseline value of 521 ± 71 μmol to a peak of $3,376 \pm 470.4$ $\mu\text{mol/24 h}$ on day 7 ($P < 0.001$) in five patients with RCC. By day 11, when the second course of IL-2 was started, NO_3^- excretion returned to 637.5 ± 72.85 $\mu\text{mol/24 h}$, a value close to the control level. As was observed during the first IL-2 course, NO_3^- excretion increased during the second IL-2 infusion, again peaking ($2,014.9 \pm 608.5$ $\mu\text{mol/24 h}$; $P < 0.05$) 2 d after completion of therapy. Thereafter, NO_3^- excretion returned toward baseline. The consolidation course of IL-2 or IL-2 plus LAK cell therapy produced a significantly smaller increase in 24-h mean urine NO_3^- output than the induction course ($P < 0.001$).

In eight treatment courses in seven patients with MM (patient 5 had two complete courses of IL-2 therapy separated by a 3-mo interval), the excretion of NO_3^- increased significantly from baseline (600 ± 80 $\mu\text{mol/24 h}$) to $1,372 \pm 271$ $\mu\text{mol/24 h}$ during the first 24 h of IL-2 therapy ($P < 0.05$) and stabilized at this level until day 6 when urine NO_3^- excretion rose ($3,368 \pm 1,041$ and $5,401 \pm 1,436$ $\mu\text{mol/24 h}$ on days 6 and 7, respectively [$P < 0.001$]). The mean 24-h output of NO_3^- then fell gradually to near baseline level (766 $\mu\text{mol/24 h}$) by day 11. The highest 24-h urine excretion of NO_3^- by an individual patient was $11,824$ μmol on day 7. After reinstitution of IL-2 with and without LAK cell administration (days 11–17), urine NO_3^- again increased (mean values of $2,041$ $\mu\text{mol/liter}$ on day 16 and $2,670$ $\mu\text{mol/liter}$ on day 17) but did not reach levels observed during the priming course of IL-2 ($P < 0.003$). The differences in 24-h urine NO_3^- output and serum NO_3^- levels between the induction and consolidation courses of IL-2 could be related, in part, to the fact that both MM and RCC patients received fewer infusions of IL-2 during the consolidation course because of more severe toxic side effects. MM and RCC patients received a mean of 12.5 and 10.6 priming IL-2 infusions and 8.5 and 5 consolidation infusions of 14 possible doses in each treatment course (Table I).

Effect of IL-2-induced changes in renal function on serum NO_3^- levels and 24-h urine NO_3^- excretion. The data were further analyzed in order to determine whether the IL-2-induced increase in serum NO_3^- resulted from a primary rise in NO_3^- production or from decreased renal NO_3^- excretion (see Fig. 2, panels c, d, e). This distinction was important since IL-2 administration caused reproducible decreases in creatinine clearance that could have contributed to NO_3^- retention and thus elevation of serum NO_3^- levels (36, 37). The data summarized in Fig. 2 (left) demonstrate that the observed increases in serum NO_3^- levels of RCC patients resulted from a primary rise in NO_3^- production and not from a major decrease in renal NO_3^- excretion. This is concluded because the increases in serum and urine NO_3^- (panels a and b) were accompanied by parallel elevations in serum $\text{NO}_3^-/\text{creatinine}$ (threefold increase; $P < 0.005$), serum $\text{NO}_3^-/\text{blood urea nitrogen (BUN)}$ (eightfold increase; $P < 0.001$) and urine $\text{NO}_3^-/\text{creatinine}$ (fivefold increase; $P < 0.02$) ratios (panels c through e). In MM patients

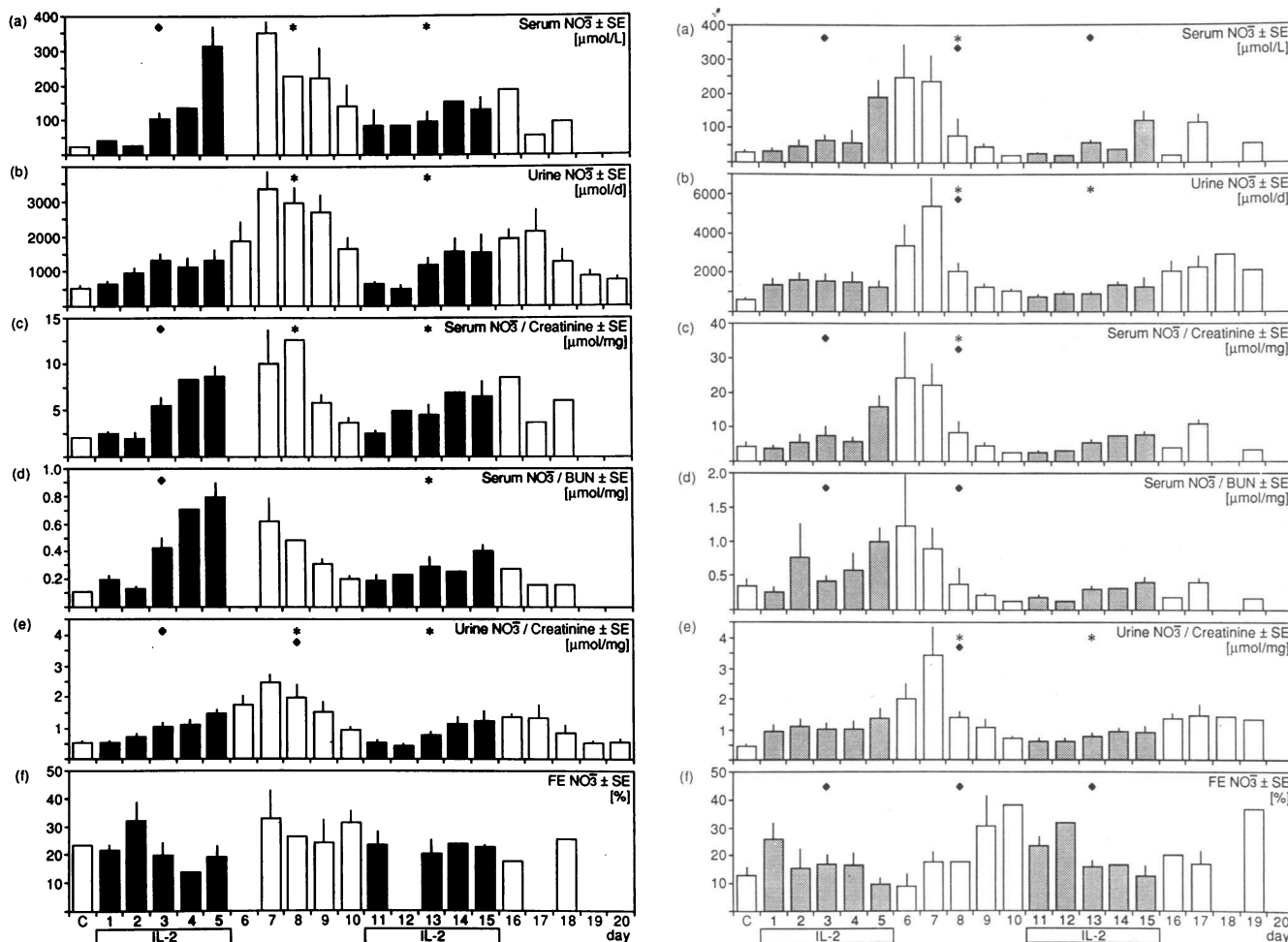


Figure 2. Serum NO_3^- levels and renal NO_3^- handling in (left) RCC patients ($n = 5$) and (right) MM patients ($n = 7$) treated with two courses of IL-2. Open bars denote control days and the two recovery periods after the discontinuation of IL-2. Closed bars denote days of IL-2 administration (induction and consolidation courses). Values are expressed as means $\pm$ SEM. Diamond (♦) indicates that the quantitative change of a depicted variable during IL-2 therapy (induction and consolidation courses) and during interruption or termination of IL-2 therapy is significant (at least $P < 0.05$), or in other words that the slope of a given change is significantly different from "zero." Asterisk (*) indicates that values measured during a given IL-2 or recovery period are significantly different (at least $P < 0.05$) from those in the immediately preceding treatment or recovery period, respectively. L, liter.

(Fig. 2, right) serum NO_3^- (panel a) and urinary NO_3^- excretion (panel b) also increased in parallel with the ratios of serum NO_3^- /creatinine (panel c; $P < 0.005$), serum NO_3^- /BUN (panel d; $P < 0.003$) and urine NO_3^- /creatinine (panel e; $P < 0.004$) ratios. The significant rise of the former two ratios indicates that the elevation in serum NO_3^- exceeded simultaneous increases in creatinine and BUN induced by IL-2. The portion of filtered NO_3^- that is excreted, remained relatively unchanged in RCC patients as evidenced by the fractional excretion (FE) of NO_3^- (Fig. 2, left, panel f). The pattern of the FE NO_3^- for MM differed somewhat from that observed for the RCC patients. Fig. 2, right, panel f, shows that the fractional excretion of NO_3^- fell significantly during both courses of IL-2 therapy and rose during the first recovery period. These results suggest that marked IL-2-induced generation and modest NO_3^- retention contributed to the overall rise in serum NO_3^- levels.

Origin of NO_3^- synthesized in response to IL-2 treatment in humans. Inorganic nitrogen oxides synthesized by cytokine-treated rodent macrophages are derived from a terminal guan-

dino nitrogen atom of L-arginine (see Fig. 1). In order to determine whether NO_3^- synthesized by humans in response to IL-2 originated from the same precursor molecule, we administered intravenous L-[^{15}N]arginine to two patients. The synthesis of [^{15}N] NO_3^- from L-[^{15}N]arginine was compared to that of [^{15}N] urea, a molecule known to be exclusively derived from the guanidino portion of L-arginine via the arginase reaction of the urea cycle (19). Fig. 3, panels a and b, shows the serum NO_3^- levels and the 24-h urine output of NO_3^- of patient 10 with MM who was administered intravenous L-[^{15}N]arginine. Patient 10 had the characteristic increase of IL-2-induced NO_3^- synthesis as reflected by total 24-h urine NO_3^- output and serum NO_3^- levels. L-[^{15}N]arginine (18.4 mmol) was administered to patient 10, both before (day 0) and after (day 6) the induction course of IL-2, as shown in Fig. 3, panels c-e. A total of 17.6 μmol of [^{15}N] NO_3^- and a total of 3.1 mmol of [^{15}N]urea (derived from 0.1% and 17%, respectively, of the L-[^{15}N]arginine molecules infused) were excreted in the urine during the subsequent 24-h period (day 1). The second infusion of L-[^{15}N]arginine was ad-

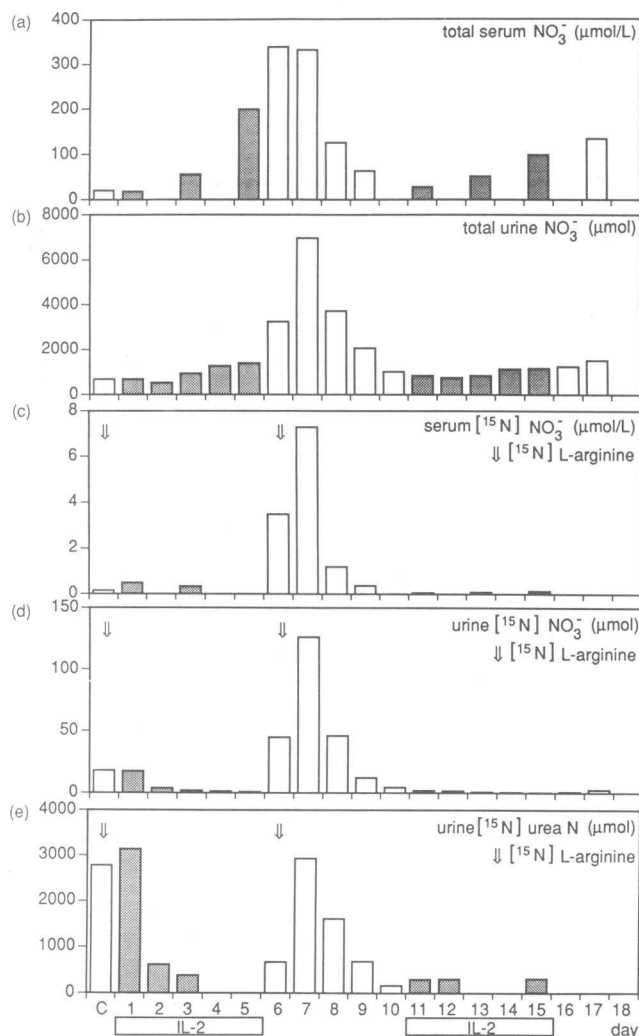


Figure 3. $[^{15}\text{N}]\text{NO}_3^-$ and $[^{15}\text{N}]\text{urea}$ excretion and serum $[^{15}\text{N}]\text{NO}_3^-$ values by patient 10 administered L- $[^{15}\text{N}_2]\text{arginine}$ HCl (arrows). See legend to Fig. 2 for an explanation of the symbols. Panels *a* and *b* show total serum NO_3^- levels and 24-h urinary NO_3^- excretion, respectively. Patient 10 received two 3.9-g (18.4 mmol) infusions of L- $[^{15}\text{N}]\text{arginine}$. The first was administered during the 24-h period before beginning induction IL-2 therapy (day 0) and again during the 24-h period immediately after terminating induction IL-2 therapy (day 6). L, liter.

ministered to patient 10 after completion of the induction course of IL-2 on day 6. There were 126 μmol of $[^{15}\text{N}]\text{NO}_3^-$ and 2.9 mmol of $[^{15}\text{N}]\text{urea}$ (derived from 0.7% and 16%, respectively, of L- $[^{15}\text{N}]\text{arginine}$ infused) excreted in the urine during the 24-h period after the L- $[^{15}\text{N}]\text{arginine}$ infusion was terminated (day 7). This represented a seven-fold increase in incorporation of ^{15}N -labeled terminal guanidino nitrogen atoms of L-arginine into urine $[^{15}\text{N}]\text{NO}_3^-$ and no change in the synthesis of $[^{15}\text{N}]\text{urea}$ after the 5-d induction course of IL-2.

The synthesis of unlabeled NO_3^- and urea-N from endogenous sources of L-arginine was compared to the synthesis of $[^{15}\text{N}]\text{NO}_3^-$ and $[^{15}\text{N}]\text{urea}$ -N from L- $[^{15}\text{N}]\text{arginine}$ infused before and after administration of the induction course of IL-2 to patient 10 (see Fig. 3). This analysis determines the percentage of total urea and NO_3^- molecules in the 24-h urine sample containing ^{15}N label derived from L- $[^{15}\text{N}]\text{arginine}$. Unlabeled 24-h

urinary urea-N increased from a pretreatment level of 321 mmol/24 h to a post-IL-2 treatment level of 430 mmol/24 h. The corresponding values for ^{15}N -labeled urea-N was 3.1 mmol on day 1 and 2.9 mmol on day 7 resulting in a ratio of ^{15}N -labeled to unlabeled urea-N of 0.97% (3.1 mmol $[^{15}\text{N}]\text{urea}$ -N/321 mmol urea-N $\times 100$) and 0.67% (2.9 mmol $[^{15}\text{N}]\text{urea}$ -N/430 mmol urea-N $\times 100$) on days 1 and 7, respectively. Corresponding measurements of urinary NO_3^- demonstrated a 10-fold increase of 24 h urine NO_3^- derived from endogenous sources of L-arginine (669 mmol NO_3^- on day 1 and 6,848 mmol NO_3^- on day 7) and a sevenfold increase of labeled $[^{15}\text{N}]\text{NO}_3^-$ (17.6 mmol on day 1 and 126.2 mmol on day 7) during IL-2 treatment. The ratio of labeled and unlabeled urinary $[^{15}\text{N}]\text{NO}_3^-$ was 2.6% (17.6 mmol $[^{15}\text{N}]\text{NO}_3^-$ /669 mmol $\text{NO}_3^- \times 100$) and 1.8% (126.2 mmol $[^{15}\text{N}]\text{NO}_3^-$ /6,848 mmol $\text{NO}_3^- \times 100$) on days 1 and 7, respectively. These results show that a similar proportion of total urea and total NO_3^- molecules excreted in the 24-h urine sample contained ^{15}N label derived from L- $[^{15}\text{N}]\text{arginine}$ (pre- and post-IL-2).

Fig. 3, panel *c*, shows serum $[^{15}\text{N}]\text{NO}_3^-$ levels in patient 10. After the pre-IL-2 treatment infusion of L- $[^{15}\text{N}]\text{arginine}$ the serum $[^{15}\text{N}]\text{NO}_3^-$ concentration reached a maximum of 0.5 $\mu\text{mol/liter}$ (day 1). However, the serum $[^{15}\text{N}]\text{NO}_3^-$ of patient 10 was 3.5 $\mu\text{mol/liter}$ (1% of 354 $\mu\text{mol/liter}$ total serum NO_3^-) 12 h after starting the L- $[^{15}\text{N}]\text{arginine}$ infusion (day 6). Immediately after terminating the L- $[^{15}\text{N}]\text{arginine}$ infusion (day 7), the serum $[^{15}\text{N}]\text{NO}_3^-$ was 7.3 $\mu\text{mol/liter}$ (2.2% of 331 $\mu\text{mol/liter}$ total serum NO_3^-) and declined thereafter.

A second patient was administered L- $[^{15}\text{N}]\text{arginine}$. The results confirmed that the IL-2-induced increase in urine and serum NO_3^- was derived from a terminal guanidino nitrogen atom of L-arginine (data not shown). In summary, the ^{15}N -labeling studies demonstrate that (a) IL-2 induces a marked increase in synthesis of NO_3^- but not urea from a terminal guanidino nitrogen atom of L-arginine, and (b) the ratio of ^{15}N -labeled molecules to unlabeled molecules remains constant regardless of whether synthesis was enhanced by IL-2 (NO_3^-) or whether synthesis remained relatively unaltered (urea).

Effects of IL-2 on peripheral vascular resistance and renal tubular function. Despite progressive declines in creatinine clearance (days 3–6; panel *a*), Fig. 4 (left) urine output of RCC patients receiving IL-2 was maintained with intravenous fluids, albumin infusions, furosemide, and dopamine as needed. Mean blood pressure (panel *e*) fell from 85 ± 4 before IL-2 therapy to a nadir of 67 ± 3 mmHg ($P < 0.004$) by day 4. This IL-2-induced decrease in creatinine clearance and blood pressure was associated with a marked increase in weight (from 69 ± 5.2 to 73.7 ± 5.7 kg on day 6 and profound reversible sodium retention (see Fig. 4, left, panel *b*, $P < 0.001$). 2 d after discontinuation of IL-2, urine volume rose strikingly (from 1.9 ± 0.5 on day 6 to 4.2 ± 0.6 liter/24 h on day 7; $P < 0.001$). This diuresis persisted until day 11, when the second course of IL-2 was begun. Predictably, body weight fell and creatinine clearance and blood pressure rose simultaneously (both $P < 0.01$). The second course of IL-2 produced a similar decrease in urine output and milder fall in blood pressure and again caused salt and water retention and thus weight gain. All alterations were readily reversible when IL-2 was discontinued. Both panels *c* of Fig. 4 illustrate the FE of urea, another variable that can be used to assess changes in tubular reabsorption. Within 3 d of beginning IL-2 therapy, FE of urea decreased modestly, rose as

IL-2 was discontinued, and fell again when IL-2 was readministered. This pattern demonstrates that IL-2 caused both enhanced Na and urea reabsorption. Panel *d* of Fig. 4 (*left*) depicts the urine to plasma ratio of creatinine. As creatinine clearance and sodium excretion declined with IL-2 therapy (days 3–5), the urine to plasma ratio of creatinine fell from 40.7 ± 4.4 to 23.3 ± 9.8 ($P < 0.003$). A further decrease to a low of 11.2 ± 3.2 occurred by day 7, a time when IL-2 had been discontinued and when creatinine clearance, blood pressure, urine volume, and salt excretion increased dramatically.

Readministration of IL-2 on day 11 raised the urine to plasma ratio of creatinine to 70.1 ± 3.0 ($P < 0.001$). Taken together, these data demonstrate that proximal renal tubular function and the urinary concentrating capacity of patients with unilateral nephrectomies were preserved and not grossly affected by IL-2.

Fig. 4 (*right*) summarizes the corresponding data from the MM patients. In general, the hemodynamic and renal responses to IL-2 administration were similar to those observed in RCC patients. It is of note, however, that virtually all functional changes were less severe when compared to those in RCC patients, whose baseline renal function was reduced because of prior unilateral nephrectomy. Finally, there was no evidence of major nephrogenous NO_3^- production in either MM or RCC patients. The FE of NO_3^- (panels *f* in Fig. 2) was

never greater than $\sim 40\%$ indicating that $\sim 60\%$ of the filtered NO_3^- was reabsorbed. Significant renal vascular or tubular NO_3^- production during IL-2 therapy would have caused the FE of NO_3^- to rise further.

Discussion

In 1981 Green and co-workers (48), while studying the biology of nitrosamine carcinogenesis, reported metabolic balance studies that showed that conventional and germ-free rats synthesize NO_3^- . This confirmed an earlier study suggesting that mammals, in addition to certain procaryotes, synthesized inorganic nitrogen oxides (49). These workers also investigated NO_3^- metabolism in long-term metabolic balance studies on healthy humans (47). During one of these studies a human subject on a low NO_3^- diet developed an unexpected increase of NO_3^- biosynthesis that occurred simultaneously with the onset of fever and diarrhea (50). Compared to baseline levels, urinary NO_3^- excretion increased ninefold during the illness. After this observation, Wagner et al. examined the effect of lipopolysaccharide (LPS) treatment on NO_3^- excretion in rats (51). The results showed that LPS caused a marked (ninefold) increase in NO_3^- excretion. Stuehr and Marletta (52) followed up these observations with experiments that demonstrated that murine macrophages cultured in vitro and exposed to LPS synthesized NO_2^- and NO_3^- . These studies defined inorganic nitrogen oxide syn-

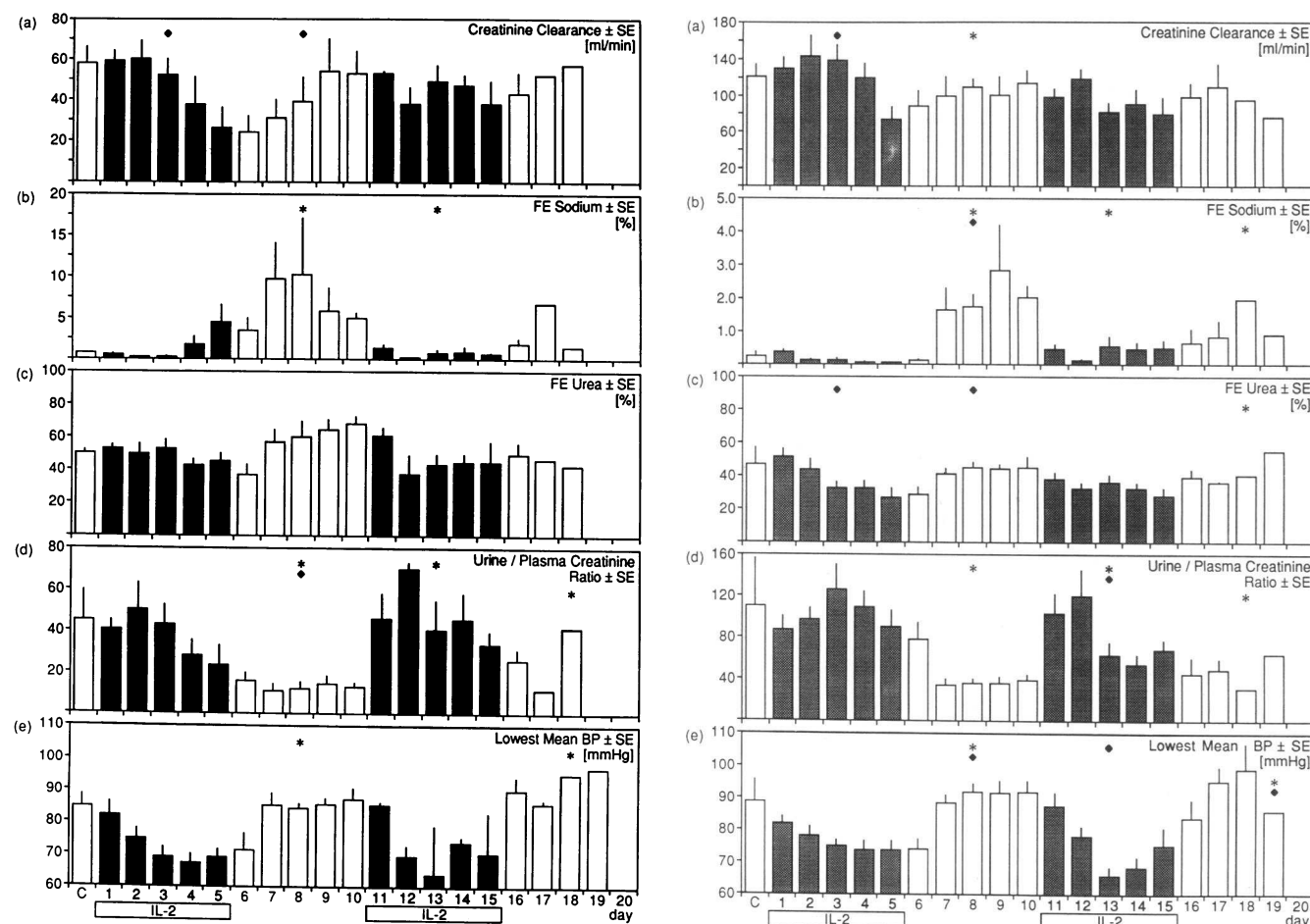


Figure 4. Renal function and blood pressure in (*left*) RCC patients ($n = 5$) and (*right*) MM patients ($n = 7$) treated with two courses of IL-2. See legend to Fig. 2 for details and explanation of the symbols.

thesis as a mammalian process but left open the question of precursor molecule identity and biological significance.

In 1987 our laboratory reported that L-arginine was the only amino acid in the culture medium required by cytotoxic activated macrophages to inhibit DNA replication and to cause inhibition of mitochondrial (4Fe-4S) enzymes in tumor target cells (11). L-Arginine was converted to NO_2^- and L-citrulline without loss of the guanidino carbon atom (12). These experiments identified L-arginine as the precursor molecule for mammalian inorganic nitrogen oxide synthesis, L-citrulline as an additional product, and established a role for the L-arginine/NO pathway as an effector mechanism of cell-mediated immunity. NO was then demonstrated to be the precursor of the NO_2^- and NO_3^- previously shown to be synthesized from L-arginine by activated macrophages (13, 15, 16). In 1987, two vascular physiology laboratories reported that NO released by endothelial cells accounted for the biological activity of endothelium-derived relaxing factor (53, 54). This was followed by a second report in 1988 that demonstrated that NO synthesized by endothelial cells is also derived from a terminal guanidino nitrogen atom of L-arginine (30). Therefore, similar L-arginine/NO pathways were shown to exist in cytotoxic activated macrophages and endothelial cells but the pathways appeared to have been adapted for different physiological functions (e.g., a cytokine-inducible isoenzyme that synthesizes large quantities of NO during cell-mediated immune responses and a constitutive isoenzyme that synthesizes small quantities of NO that functions as an intercellular message).

There is limited information to date on L-arginine/NO pathways in humans. The metabolic balance studies of Green et al. demonstrated that normal adult humans with unrestricted activity endogenously produce $\sim 690 \mu\text{mol}$ of nitrate/24 h (47). After the discovery of the L-arginine/NO pathways, this basal endogenous synthesis was explained by activity of the constitutive low-output NO synthase in maintenance of vascular tone (30), neural transmission (31–33), and in other functions such as chemotaxis (55). NO appears to be an important regulator of resting and stimulated regional blood flow in humans. N^G monomethyl-L-arginine, a potent inhibitor of both NO-synthesizing pathways (11, 12, 30), was infused into the brachial arteries of healthy volunteers to study the role of NO in the control of forearm blood flow (56). This agent caused a 50% fall in basal blood flow and attenuated the dilator response to acetylcholine but not to glycerol trinitrate. Taken together these studies provided evidence for the existence of a constitutive pathway in humans. Until now clinical studies evaluating human subjects for cytokine-inducible NO synthase activity have not been performed. We and others have not been successful in inducing high-output NO synthesis from human cells cultured in vitro by treatment protocols with cytokines that are highly effective with rodent cells (57; Hibbs et al., unpublished data). However, a recent report describes the induction of high-output nitrite synthesis by cytokine-treated human blood monocyte-derived macrophages infected with *Mycobacterium avium* (29). The reason why it is difficult to detect activity of the cytokine-inducible pathway in human cells in vitro is not clear. However, results reported in this study demonstrate that IL-2 is a potent inducer of high-output NO synthesis from L-arginine in human subjects with advanced malignancy.

There is evidence to suggest the IL-2 may not directly induce toxic effects or antitumor effects but may act as a factor that mobilizes and amplifies cell-mediated immunity by induc-

ing the synthesis of other cytokines (2–7). Elevated levels of $\text{IFN}\gamma$ and TNF have been detected in the serum of patients receiving IL-2 (2, 3, 5–7). Furthermore, the peak serum levels of these three cytokines induced by IL-2 infusions in humans occurred at the same time that we measured maximum serum NO_3^- excretion in the current study. It is of interest that $\text{IFN}\gamma$, TNF (serum concentrations) and IL-1 (elevated mRNA) measured in patients administered IL-2 (2, 3, 5–7) are known to induce the high-output NO synthase in rodents (17, 18, 58–62). Full induction of the high-output NO synthase in murine macrophages is known to require $\text{IFN}\gamma$ and a cosignal, such as LPS or TNF (17, 18). The high-output NO synthase also can be induced in nonmacrophage rodent cells including tumor cells (58, 59), fibroblasts (58, 60), hepatocytes (61), and endothelial cells (62) by $\text{IFN}\gamma$ plus TNF (IL-1 is also a cosignal in the case of nonmacrophage rodent cells [58, 60]). In human subjects, IL-2 therapy induces cytokines ($\text{IFN}\gamma$, TNF, and IL-1) known to activate the high-output NO synthase in macrophage and nonmacrophage rodent cells. Therefore, it is possible that the increased NO_3^- measured in the current study is derived from high-output NO synthesis induced by $\text{IFN}\gamma$, TNF, and IL-1 in multiple cell types that include macrophages, endothelial cells, fibroblasts, hepatocytes, and tumor cells.

Two normal human volunteers with unrestricted activity were reported to excrete 24 and 17 mmol of urinary $[\text{N}^{15}]\text{NO}_3^-$ during the 24-h period after the oral administration of L- $[\text{N}^{15}]$ -arginine (the total dose of L- $[\text{N}^{15}]$ arginine was not given) (63). This observation provides further evidence for the synthesis of NO_3^- from L-arginine in humans, probably, in this case, by the constitutive low-output NO synthase. The current study demonstrates that the marked increase of serum and urine NO_3^- induced by IL-2 administration is also derived from a terminal guanidino nitrogen atom of L-arginine. Patients were maintained on TPN ($\text{NO}_2^-/\text{NO}_3^-$ -free) or on a low- NO_3^- diet throughout the treatment course. However, the patients were administered virtual exclusive TPN during the 5-d courses of IL-2 as well as on days 6 and 7 after induction therapy (see Fig. 2) because of IL-2-associated anorexia, nausea, vomiting, and diarrhea. The use of parenteral L- $[\text{N}^{15}]$ arginine eliminated possible $[\text{N}^{15}]\text{NO}_3^-$ synthesis from oral L- $[\text{N}^{15}]$ arginine by intestinal microflora. Patient activity was confined to a hospital room (bed rest) which minimized any possible effects of exercise related vasodilation. The results of our study show increased incorporation of terminal guanidino nitrogen atoms of L-arginine into NO_3^- during IL-2 therapy. This increased incorporation of ^{15}N into NO_3^- without increased incorporation into urea is strong evidence for the existence of a human cytokine-inducible high-output NO synthase.

For our small series of patients, the clinical response to IL-2 therapy could not be correlated with the pattern of NO_3^- production. Further work is required to determine whether activity of the cytokine-inducible high-output L-arginine/NO pathway contributes a neutral, positive, or negative effect to IL-2-mediated tumor regression. However, NO must be considered a candidate effector molecule capable of causing or mediating cytostatic antitumor effects (11–13, 58). This reactive molecule may also contribute significantly to certain toxic effects observed in patients receiving IL-2 therapy, such as hypotension. This is supported by partial abrogation of IL-2-induced toxicity in mice by passive immunization against TNF (4), one of the signals required to activate the inducible NO synthase (monoclonal antibodies to TNF have a similar protective effect

against bacteremic shock [64]). Further support for this concept is derived from recent reports that NO may mediate TNF-induced vascular changes. N^G -monomethyl-L-arginine reversed TNF (65) and endotoxin (66) induced decreased peripheral vascular resistance and mean arterial pressure in dogs. These studies provide evidence for a role for NO in the pathogenesis of decreased vascular resistance and hypotension observed in LPS- or TNF-treated dogs. It also has been demonstrated recently that IL-1 induces L-arginine-dependent cyclic GMP and NO production in rat vascular smooth muscle cells (67). Therefore, it is reasonable to suggest that NO synthesis from L-arginine could play a major role in development of decreased vascular resistance and hypotension in IL-2-treated patients.

The current study provided an opportunity to evaluate renal function in humans under pathophysiological conditions that closely simulate endotoxin/LPS induced shock (hypotension and renal hypoperfusion) in a clinical setting associated with elevated levels of TNF in tissues. The data are consistent with the suggestion made by Marsden and Ballermann (68) that TNF-induced NO produced within the renal parenchyma actually protects against development of acute tubular necrosis by maintaining postglomerular circulation. This is also consistent with the observation that synthesis of NO from L-arginine lessens hepatic damage in a murine model of endotoxin-induced shock (69). NO reduces renal vascular resistance and inhibits platelet activation (aggregation and adhesion [70, 71]). Thus NO has the potential to promote maintenance of renal perfusion under normal and pathological conditions (30, 53, 54, 70–74). It is likely that cyclic GMP, a second messenger induced by NO, is elaborated by the kidney during IL-2 therapy (68, 73, 74). It has been shown that atrial natriuretic factor, a peptide with vasodilating and diuretic/natriuretic properties, ameliorates, via cyclic GMP, the severity of acute renal failure (75, 76).

The reversible form of IL-2-induced acute renal failure differs significantly from that observed in endotoxemic patients and animals (77–82). In the latter, acute and sometimes irreversible tubular necrosis is common. Specifically patients with endotoxin-induced acute tubular necrosis lose their capacity to retain salt (high FE of sodium) and to concentrate their urine (low urine to plasma creatinine ratio). These alterations were not observed in the IL-2-treated patients evaluated in this study (see Fig. 4). Since IL-2-treated patients resemble patients with endotoxemia in many regards (elevated TNF levels, hypotension, elevated cytokine levels, etc.), it is of interest that acute tubular necrosis is a rare consequence of IL-2 therapy. This observation is even more surprising since all patients received indomethacin, an agent that predisposes to the development of acute tubular necrosis (83). In conclusion, the results reported here show highly significant NO_3^- synthesis in patients receiving IL-2 therapy for advanced malignancy, and demonstrate that a cytokine-inducible high output L-arginine/NO pathway exists in humans.

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