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AUSTRALIA

PATENTS ACT 1990

NOTICE OF ENTITLEMENT

We, **Board of Regents, The University of Texas System** AND **Cornell Research Foundation, Inc.**, the applicants/Nominated Persons in respect of Application No. 28662/92 state the following:-

The Nominated Persons are entitled to the grant of the patent because the Nominated Person, Board of Regents, The University of Texas System derives title to the invention from inventor Robert G. Kilbourn by assignment and the Nominated Person, Cornell Research Foundation, Inc. derives title to the invention from the inventors Steven S. Gross and Owen W. Griffith by assignment.

The Nominated Persons are entitled to claim priority from the applications listed in the declaration under Article 8 of the PCT because the Nominated Persons are the assignees of the applicants in respect of the applications listed in the declaration under Article 8 of the PCT, and because those applications were the first applications made in a Convention country in respect of the invention.

DATED this EIGHTEENTH day of MARCH 1994

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a member of the firm of
DAVIES COLLISON
CAVE for and on behalf
of the applicant(s)

(DCC ref: 1651976)

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AMINO ACIDS CONTAINING PARENTERAL FORMULATIONS FOR THE TREATMENT OF HYPOTENSION AND RELATED PATHOLOGIES

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(71) Applicant(s)
BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM; CORNELL RESEARCH FOUNDATION, INC.

(72) Inventor(s)
ROBERT G. KILBOURN; OWEN W. GRIFFITH; STEVEN S. GROSS

(74) Attorney or Agent
DAVIES COLLISON CAVE , 1 Little Collins Street, MELBOURNE VIC 3000

(57) Claim

1. A method for maintaining an animal's blood pressure at a physiologically acceptable level, the method comprising administering to an animal an arginine-free composition comprising:

alanine;
proline;
serine;
leucine;
methionine;
phenylalanine;
lysine;
valine
isoleucine;
threonine;
tryptophan;
histidine;
tyrosine;
glycine; and
aspartic acid.

.../2

0.25-0.75 g/l tyrosine;

4-5 g/l glycine; and

2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

21. A method of providing nutritional support to an animal at risk for hypotension comprising administering a nitric oxide synthase inhibitor and an arginine-free mixture of amino acids wherein the arginine-free mixture of amino acids comprises:

3-4 g/l isoleucine;

4-6 g/l leucine;

3-4 g/l lysine;

1-2 g/l methionine;

1-2 g/l phenylalanine;

2-3 g/l threonine;

0.5-1.5 g/l tryptophan;

3-4 g/l valine;

4-5 g/l alanine;

1-2 g/l histidine;

3-4 g/l proline;

1-2 g/l serine;

52. A method for treating septic shock related hypotension in an animal exposed to endotoxin comprising administering an arginine-free mixture of amino acids and either an interleukin-1 receptor antagonist or an anti-endotoxin antibody wherein the amino acids comprise:

3-4 g/l isoleucine;

4-6 g/l leucine;

3-4 g/l lysine;

1-2 g/l methionine;

1-2 g/l phenylalanine;

2-3 g/l threonine;

0.5-1.5 g/l tryptophan;

3-4 g/l valine;

4-5 g/l alanine;

1-2 g/l histidine;

3-4 g/l proline;

1-2 g/l serine;

0.25-0.75 g/l tyrosine;

4-5 g/l glycine; and

2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient and wherein said antagonist or antibody is administered concurrently with or after the arginine-free mixture of amino acids.

CORRECTED VERSION *

PCT

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28662/92

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(71) Applicants: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US). CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Dr., Suite 105, Ithaca, NY 14850 (US).

(72) Inventors: KILBOURN, Robert, G. ; 5490 Braes Valley, Apt. 408, Houston, TX 77096 (US). GRIFFITH, Owen, W. ; 430 E. 63rd Street, Apt. 6H, New York, NY 10021 (US). GROSS, Steven, S. ; 176 Broadway, New York, NY 10038 (US).

(74) Agent: MAYFIELD, Denise, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: AMINO ACIDS CONTAINING PARENTERAL FORMULATIONS FOR THE TREATMENT OF HYPOTENSION AND RELATED PATHOLOGIES

(57) Abstract

An anti-hypotensive formulation comprising an essentially arginine-free or low arginine (less than about 0.1 %, most preferably, about 0.01 %) containing mixture of amino acids is provided. The formulations may further include ornithine, citrulline or both. Combination regimens of the described TPN solutions and an arginine analog, or the TPN formations with an anti-endotoxine antibody, an interleukin-1 receptor antagonist, or an anti-tumor necrosis antibody are also provided. Methods for prophylaxis and treatment of systemic hypotension in employing these combination regimens are also provided. A method for treating hypotension caused by nitric oxide synthesis, through administering a low or essentially arginine-free parenteral formulation is described. Methods for treating an animal in septic shock are also disclosed. Prophylaxis or treatment of systemic hypotension, particularly that hypotension incident to chemotherapeutic treatment with biologic response modifiers, such as tumor necrosis factor or interleukin-1 or -2, may be accomplished through the administration of the defined anti-hypotensive formulations or combination regimens with a TPN formulation until physiologically acceptable systolic blood pressure levels are achieved in the animal. Treatment of an animal for septic shock induced by endotoxin may also be accomplished by administering to the animal the arginine-free formulations described. Methods for providing nutritional support for an animal at risk of or in septic shock may also be provided through the administration of an arginine-free formulation or the combination regimens.

DESCRIPTION

AMINO ACIDS CONTAINING PARENTERAL FORMULATIONS FOR THE TREATMENT OF HYPOTENSION AND RELATED PATHOLOGIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of prophylaxis and treatment of hypotension. Most particularly, the present invention proposes methods for the control and inhibition of hypotension or systemic shock through the administration of particularly defined formulations and combination regimens with limited nitric oxide- generating potential. The present invention also relates to specially tailored nutritional formulations for patients at risk of hypotension or systemic shock which include low concentrations of arginine or are arginine-free. Particular embodiments of the formulations also include ornithine or citrulline as urea cycle substrates. Anti-hypotensive TPN formulations for the general nutritional support of patients are also provided. The invention also relates to combination therapeutic regimens, particularly the combination of nitric oxide inhibitors and parenteral formulations. The present invention also relates to the field of arginine analogs, anti-tumor necrosis factor antibodies, anti-endotoxin antibodies and interleukin-1 receptor antagonists as part of a therapeutic regimen for the treatment of hypotension, septic shock and related conditions.

2. Background of the Art

Hypotension, or low blood pressure, is a complicating and often life-threatening condition attendant to shock, traumatic injury, sepsis, the administration of immunomodulators, as well as other

situations. Thus, the risk of hypotension affects a significant number of persons throughout the world. For example, septic shock, a life-threatening complication of bacterial infections, affects 150,000 to 300,000 patients annually in the United States alone.[1]

The cardiovascular collapse and multiple metabolic derangements associated with septic shock are due largely to bacterial endotoxin (ET), which has been shown to elicit a septic shock-like condition when administered to animals[2]. ET is known to stimulate the synthesis and release of several cytokines and biological mediators having hypotensive activity; among the factors released, TNF, platelet activating factor (PAF), prostacyclin and complement-derived C5a anaphylatoxin have been proposed as contributors to the cardiovascular collapse of septic shock[3-6].

Although it has been shown that animals pretreated with anti-TNF antibodies[7], PAF receptor antagonists[8], and prostacyclin synthesis inhibitors[9] are significantly protected against septic shock, the relative importance of these mediators in the pathology of septic shock is presently uncertain.

There is also evidence that some of these mediators may act indirectly *via* release of secondary mediators. Thus, the finding that anti-TNF antibodies have little or no protective effect when given after ET exposure[7] suggests that TNF stimulates the production of another factor that is the actual hypotensive agent. Once initiated, synthesis and release of that factor can apparently continue even in the absence of detectable TNF. In 1980, Furchgott *et al.* (1980)[10] demonstrated that endothelial cells, which line blood vessels, can be stimulated to release a substance which relaxes vascular smooth muscle (i.e., causes vasodilatation). Since the

chemical nature of this substance was completely unknown, it was simply named endothelium-derived relaxing factor (EDRF). It is hypothesized that many naturally-occurring substances which act as physiological vasodilators mediate all or part of their action by stimulating release of EDRF; these substances include, acetylcholine, histamine, bradykinin, leukotrienes, ADP, ATP, substance P, serotonin, thrombin and others.

The extremely short lifetime of EDRF (several seconds) hampered early efforts to chemically identify this molecule. In 1987, several laboratories suggested that EDRF may be nitric oxide (NO), which spontaneously decomposes to nitrate and nitrite. However, the fundamental problem in accepting this NO hypothesis was that mammalian systems were not known to contain an enzymatic pathway which could synthesize NO; additionally, a likely precursor for NO biosynthesis was unknown.

After observing that the arginine analog L-N^G-methylarginine (L-NMA) could inhibit vascular EDRF/NO synthesis induced by acetylcholine and histamine, and that EDRF/NO synthesis could be restored by adding excess L-arginine, certain of the present inventors proposed that arginine is the physiological precursor of EDRF/NO biosynthesis[11]. Certain of the present inventors later demonstrated that inhibition of EDRF/NO synthesis in the anesthetized guinea pig raises blood pressure.

The enzyme responsible for NO synthesis (nitric oxide synthase) has been partially characterized by some of the present inventors[14] and acts to oxidize a terminal nitrogen of the quanidino group of arginine, resulting in production of nitric oxide and citrulline. Macrophage-derived nitric oxide is now considered an important tumoricidal and bactericidal agent.

It has been reported that macrophage cells become "activated" by 12-36 hour after treatment with gamma-interferon, bacterial endotoxin and various cytokines *in vitro*. However, this *in vitro* "activation" system had been associated only with the initiation of tumor cell killing.

However, none of the literature or studies available prior to the present inventors work associated hypotension with nitric oxide, or the involvement of macrophages with hypotension.

Macrophages are a quantitatively insignificant component of normal blood vessel walls, and have never been shown to play any role in blood pressure regulation; i.e., there existed no biochemical, physiological or immunological data to suggest that macrophages had any role in pathological hypotension. Thus, the inventors sought to investigate the role of nitric oxide in systems relevant to the manifestation of hypotension, specifically the role of cytokine induced pathological hypotension, particularly on cells which comprise blood vessel walls.

Cytokines are well known to cause morphological and functional alterations in endothelial cells described as "endothelial cell activation". Distinct immune-mediators such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and gamma-interferon (IFN) appear to induce different, but partially overlapping, patterns of endothelial cell activation including increased procoagulant activity, PGI2 production, HLA antigen expression[18] and lymphocyte adhesion molecule activation. Although these cytokines are reported to cause hypotension, vascular hemorrhage, and ischemia, the underlying mechanisms of altered vasoactivity are unclear.

In both clinical and animal studies on the effects of biological response modifiers, a major dose limiting toxicity has been hypotension and vascular leakage. The inventors have observed that endotoxin and tumor necrosis factor can induce over production of nitric oxide in animals. Nitric oxide is a vasoactive substance which controls resting blood pressure. This led the present inventors to postulate that hypotension in humans resulting from administration of biological response modifiers or from the development of overwhelming bacterial infections is due to excessive production of nitric oxide in sufficient concentration to relax vasoconstriction. However, macrophages are known to compose quantitatively only an insignificant component of normal blood vessel walls. Moreover, as a practical matter, it was unlikely that the amount of nitric oxide generated by circulating macrophages would be sufficient to elicit a "hypotensive" effect physiologically, as nitric oxide is not produced in vast enough quantities by the limited number of macrophages in blood vessel walls to produce such a pronounced physiological response. This, together with the recognized short half-life nitric oxide *in vivo* (3-5 seconds), opposed the theory that macrophage-derived nitric oxide was involved in hypotension.

The inventors observed that nitric oxide is derived from the amino acid L-arginine. L-arginine is a typical ingredient in commercially available TPN (total parenteral nutrition) formulations.

A TPN regimen of low or essentially arginine-free formulations is proposed by the inventors to reduce, if not eliminate, the risk of hypotension and septic shock in patients with bacterial infections. Clinical regimens which typically require the administration of a TPN formulation include, for example, nutritional support of

cancer patients and others who have no or limited ability to tolerate oral feeding.

Interleukin-1-α (IL-1) has been shown to enhance the restoration of peripheral blood leukocytes in mice myelosuppressed by cytotoxic chemotherapeutic agents.[16] In addition, IL-1 inhibits growth of murine tumors *in vitro* and *in vivo* and is cytocidal for several tumor cell lines.[17,18] Clinical trials in conjunction with the National Cancer Institute are currently underway to assess the efficacy of IL-1 as an immunorestorative agent in cancer patients exhibiting myelosuppression secondary to chemotherapy. In these trials, the does-limiting side effect of IL-1 is hypotension,[19-21] a complication that may prevent the administration of therapeutically effective doses of IL-1.

The association of IL-1 with compromised cardiovascular function is well documented in both animal and patient studies. thus, Dinarello *et al.*[22] have shown that administration of IL-1 causes a shock-like syndrome in rabbits, an effect they attributed to the overproduction of cyclooxygenase products. These authors also have shown a synergistic association between exogenous IL-1 and tumor necrosis factor (TNF) in causing shock. There is now considerable evidence that shock due to exposure to endotoxin (i.e., septic shock) is associated with increased endogenous production of both TNF and IL-1.[23] The critical role of IL-1 in endotoxic shock is substantiated by recent reports showing that administration of a human recombinant IL-1 receptor antagonist improves survival and prevents the development of hypotension in rabbits given endotoxin.

The inventors have previously shown that pathologic overproduction of nitric oxide (NO·) -- initially characterized as endothelium-derived relaxing factor[10,11] --

mediates the hypotension caused by the administration of TNF to dogs.[26]

NO· is a short-lived but potent vasodilator formed enzymatically by oxidation of one of the two equivalent guanidino (i.e., omega) nitrogens of L-arginine in a reaction catalyzed by nitric oxide synthase. Several N$^{\omega}$-substituted arginine analogues are effective inhibitors of nitric oxide synthase, and some have been shown to inhibit NO· production *in vivo*.

Some of these N$^{\omega}$-substituted arginine analogs include N$^{\omega}$-methyl-L-arginine (NMA), NNA (N$^{\omega}$-nitro-L-arginine) and NAA (N$^{\omega}$-amino-L-arginine). Some enthusiasm has been generated in regard to the use of nitric oxide inhibitors, such as NMA, as antihypotensive agents. However, this prospect has been tempered by the recent demonstration that very large doses of NMA demonstrates an increase in mortality in endotoxic rodents, while other studies have indicated that NMA increases hepatic damage following administration of endotoxin to rats. The inventors own work has also shown that treatment with large doses of arginine analogs, such as NMA results in lower cardiac output *in vivo* while increasing blood pressure, the results of which could compromise oxygen delivery to critical organs. Therefore, methods and regimens which could optimize the potential normotensive action of these agents at lower doses would make these agents significantly more acceptable and useful in the clinical setting.

A method would maximize the beneficial anti-hypotensive effects of low physiological arginine concentrations, for example, by the maintenance of an animal, on an arginine-free dietary support (TPN), coupled with moderate doses of a nitric oxide synthase inhibitor would provide a significant advantage in

managing an animal with hypotension or at risk of developing hypotension.

SUMMARY OF THE INVENTION

The present invention relates to methods and formulations for preventing or inhibiting systemic hypotension in an animal induced by a biological response modifier or by bacterial sepsis. By way of example, the biological response modifiers which are linked to causing systematic hypotension include the cytokines IFN, TNF, IL-1 and IL-2. The claimed method involves administering, preferably parenterally, an amount of a formulation which is arginine-free to a hypotensial or potentially hypotensive animal to reduce the level of serum arginine systemically, and thereby effect a reduction in the synthesis of nitric oxide.

The inventors have observed that nitric oxide is derived from the amino acid L-arginine. Therefore, the methods of the present invention include formulations having only very low concentrations of arginine or that are preferably essentially arginine-free. Reduced arginine will serve to reduce nitric oxide synthesis and thus reduce the systemic hypotension manifest in an animal with an elevated level of nitric oxide.

Particular embodiments of the TPN anti-hypotensive formulations include the addition of citrulline and/or ornithine. Citrulline and/or ornithine, which do not directly result in nitric oxide production, may be included to satisfy metabolic requirements such as those of the urea cycle, for example. As used in the present application, the term "low" arginine refers to a TPN solution which includes less than 0.1% arginine in the feeding solution (<100 mg. arginine/100 ml TPN). An even more preferred embodiment of the formulation includes a

concentration of arginine between 0.1% and 0.001% arginine. Even more preferably, the anti-hypotensive TPN of the present invention includes about 0.001% (1 mg/100 ml TPN) arginine. In a most preferred embodiment of the present methods and formulations, arginine is included in the formulation at trace amounts of less than 0.1 mg/100 ml (about 0.0001%). The formulation in its most preferred embodiment is essentially arginine-free.

In a most preferred embodiment of the described methods, the formulation employed is an essentially arginine-free formulation and is also essentially ornithine-free and citrulline free. Alternatively, the essentially arginine-free formulation may include ornithine or citrulline. So formulated, the present methods also provide a specially tailored total parenteral nutrition (TPN) formulation for a patient at risk of systemic sepsis or suffering from other nitric oxide-mediated hypotension.

Although administration of the formulation to an animal is preferably parenteral, it is contemplated that other administration routes, such as by oral administration, for example, may prove useful as the route of administration.

In one embodiment of the method, the arginine-free formulation is administered to an animal which may develop, is possibly developing, or is experiencing NO-mediated systemic hypotension. The arginine-free formulations of the present inventive methods preferably include any pharmaceutically acceptable addition salts as commensurate with planned treatments.

A particular preferred use of the method of the present invention is in the prophylaxis or treatment of systemic hypotension manifest in a patient receiving

chemotherapeutic agents, such as TNF, IL-2 (interleukin-2), IL-1 (interleukin-1), or a combination thereof. In this respect, the method involves administering to the patient, a nutritionally supportive amount of a low arginine or essentially arginine-free amino acid TPN formulation for a period of time until an elevation in the animal's systolic blood pressure to a physiologically acceptable level is demonstrated. By way of example, a physiologically acceptable systolic blood pressure level in an adult human is 100 mm Hg (100 millimeters of mercury) or greater. An adult human having a systolic blood pressure level of less than 100 mm Hg is defined as manifesting the condition known as "hypotension" for purposes of the methods of treatment defined herein. Most preferably, the formulation is prepared so as to be suitable for administration as a parenteral formulation to a patient.

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An additional important application of the present invention is as a method for the treatment of septic shock, particularly that septic shock induced by bacterial endotoxin. Although prophylaxis is not practical here, treatment to improve and eliminate the condition is essential. The preferred method of treatment of septic shock according to the present invention therefore comprises maintaining the patient on an arginine-free or low arginine formulation comprising a mixture of amino acids until the patient demonstrates a maintained systolic blood pressure within physiologically acceptable levels. In an adult human, a physiologically acceptable systolic blood pressure is at least about 100 mm Hg (100 millimeters of mercury). Most preferably, the formulation is prepared so as to be suitable for administration as a parenteral formulation, and thus must be of a physiologically acceptable pH for administration parenterally.

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arginine or essentially arginine-free formulation which includes a mixture of amino acids in nutritionally supportive concentrations. Nutritionally supportive concentrations of amino acids as included within a parenteral formulation are provided at Table 3. The mixture of amino acids is ornithine-free and citrulline-free in one particularly preferred embodiment.

A mixture of particular essential and non-essential amino acids are included in the claimed anti-hypotensive formulations. A classification of amino acids recognized by those of skill in the art as "essential" or "non-essential" is provided in Lehninger et al.'s *Biochemistry* text,[26] and in Wagner [27]. These references are specifically incorporated herein by reference for this purpose.

Studies conducted by the inventors also demonstrated that particular analogs of arginine (arginine antagonists) inhibit hypotension *in vivo*. The administration of particular arginine analog antagonists has been found by the inventors to affect a variety of biochemical pathways physiologically, most notably by decreasing the production of nitric oxide, and thus eventually the hypotensive/normotensive state in the animal.

In a more general sense, the present invention may relate to a method for the prophylaxis or treatment of nitric oxide-induced systemic hypotension in a patient requiring total parenteral nutritional support. The necessity for maintaining a patient on a parenteral nutritional regimen occurs when a patient is unable to swallow, such as is typical of patients receiving chemotherapeutic agents which induce nausea, emesis or anorexia.

As part of such a regimen, the arginine-free formulation is to be administered on an "as needed" basis, as determined by the attending physician until a sustained systolic blood pressure of at least 100 mm Hg is detectable in the patient.

While not intending to be limited to any particular theory or mechanism of action, it is postulated that the administration of an essentially arginine-free parenteral formulation will inhibit hypotension because such a reduction in serum-arginine levels will elicit a reduction in nitric oxide synthesis. Nitric oxide, as already discussed, has been observed by the inventors to constitute the vasoactive substance which causes hypotension in animals receiving biological response modifiers or endotoxin which causes septic shock. This theory is drawn from the inventors observations that patients receiving tumor necrosis factor also exhibit elevated blood nitric levels (a stable breakdown product of nitric oxide) when hypotension is manifest. The formulation may also be supplemented with ornithine, citrulline, or both. Employing the herein disclosed methods and formulations, those persons who may have already lapsed into a state of septic shock, or who are already severely ill and being maintained on a respirator with total parenteral nutritional support, may be nutritionally supported without increasing the risk of exacerbating an already existing condition of septic shock or hypotension.

Within this specification, the acronym "NO" will be understood to represent nitric oxide as well as any other additional vasoactive nitrogen oxides. In still another embodiment of the invention, the therapeutic regimen comprises administering to a subject a therapeutically effective amount of an arginine-free parenteral formulation and administering a therapeutically effective

amount of a nitric oxide synthase inhibiting arginine analog.

The arginine analogs of the present invention may be administered concurrently with or subsequent to the administration of the parenteral formulation. Most preferably, the parenteral formulation is administered, in an amount sufficient to reduce physiological concentrations of arginine prior to the administration of the arginine analog.

While any number of arginine analogs having an anti-hypotensive effect may be used in conjunction with the described therapeutic regimen, those arginine analogs most preferred include N^{ω}-methyl-L-arginine, N^{ω}-amino-L-arginine, N^{ω}-nitro-L-arginine, or a mixture thereof. Most preferably, the arginine analog of choice for use in the described therapeutic regimen is N^{ω}-methyl-L-arginine or N^{ω}-amino-L-arginine. The therapeutically effective amount of these particular analogs may be defined as between about 0.1 mg/kg and about 100 mg/kg. In a more narrowly defined aspect of the invention, the therapeutically effective amount of N^{ω}-methyl-L-arginine is a dose of between about 10 mg/kg and 30 mg/kg. The dose of arginine analog, such as N^{ω}-methyl-L-arginine most preferred is about 20 mg/kg. These dose ranges may also be used for other arginine analogs with nitric oxide inhibiting activity in the practice of the present invention.

The arginine-free parenteral formulation of the described therapeutic regimen may be defined further as including a mixture of essential and nonessential amino acids. This mixture of essential and nonessential amino acids include thse described *infra*. Again, the parenteral formulation of the therapeutic regimen is arginine-free. The pharmacologically acceptable

excipient of the parenteral formulation is most preferably a Ringers solution or saline. Of these, saline is the most preferred excipient. Ornithine (about 1-2 g/l) and/or citrulline may be added to the formulation to enhance and maintain metabolic requirements of the urea cycle in the animal.

While the arginine analogs of the therapeutic regimen may be administered according to any administration route known to those of ordinary skill in the medical art, such as through oral, parenteral or enteral routes, the arginine analog is most preferably administered *via* a parenteral route, such as by an IV, IP or subcutaneous administration. Of these, intravenous administration of the arginine analog is most preferred, particularly through IV bolus treatment.

The parenteral formulation is to be administered in an amount sufficient to reduce plasma concentrations of arginine in the animal, most preferably to an amount less than normal physiological levels. For example, the amount of the parenteral formulation sufficient to reduce plasma concentrations of arginine in an animal may constitute a continuous intravenous feeding of the formulation described herein (arginine-free) for at least 2 hours prior to administration of the arginine analog. In a most preferred aspect of the regimen, the arginine analog is administered *via* an intravenous route to the animal after an initial parenteral feeding has been established. Alternatively, the arginine analog may be administered as a separate treatment concurrently with establishment of the animal on the above-described parenteral formulation.

In still another aspect of the invention, a method for treating hypotension in an animal is provided. In one embodiment, this method comprises identifying an

animal having a systolic blood pressure of less than about 100 mm Hg, administering to said animal an anti-hypotensive parenteral formulation comprising an arginine-free mixture of essential and nonessential amino acids, administering currently with the formulation or subsequent to the formulation a therapeutically effective amount of an arginine analog capable of inhibiting nitric oxide, monitoring the blood pressure of said animal over a period of at least 24 hours, and maintaining the animal on the formulation and the arginine analog until a systolic blood pressure of at least about 100 mm Hg is detected. Detecting and monitoring blood pressure in an animal having a systolic blood pressure of less than 100 mm Hg may be achieved using standard blood pressure monitoring techniques, such as use of a blood pressure cuff for measurement of blood pressure in a human, for example, or though use of an indwelling arterial catheter connected to a pressure transducer (known to those of skill in the medical arts as an "arterial line").

According to the described method, the arginine analog is most preferably N^{ω}-methyl-L-arginine, N^{ω}-amino-L-arginine, N^{ω}-nitro-L-arginine or a mixture thereof. Most preferably, the arginine analog of choice to be used in conjunction with the described method is N^{ω}-methyl-L-arginine. The anti-hypotensive parenteral formulation of the method most preferably comprises the concentrations of essential and nonessential amino acids described *infra*.

Where the arginine analog of choice is N^{ω}-amino-L-arginine, the therapeutically effective concentration of the analog may preferably constitute a dose of between about 0.1 mg/kg to about 100 mg/kg. An even more preferred concentration range of the arginine analog, N^{ω}-amino-L-arginine, is between about 10 mg/kg to about 30 mg/kg. Even more preferably, the therapeutically

effective concentration of the arginine analog N$^{\omega}$-amino-L-arginine is about 20 mg/kg. According to a most preferred embodiment of the described method, the arginine analog is to be administered intravenously, such as in a single bolus dose.

In still another aspect of the claimed invention, a method for treating chemotherapeutic agent-related hypotension in an animal receiving a chemotherapeutic agent is provided. This method in one embodiment comprises monitoring an animal receiving a chemotherapeutic agent for a decrease in systolic blood pressure to less than 100 mm Hg to detect an animal with systemic hypotension, treating the animal with a therapeutic regimen comprising an arginine-free parenteral formulation concurrently with or followed by the administration of a therapeutically effective concentration of an arginine analog capable of inhibiting nitric oxide synthase, and maintaining the animal on the therapeutic regimen until an increase of systolic blood pressure to at least about 100 mm Hg is detectable.

Detecting and monitoring blood pressure in an animal may be accomplished using those techniques well known to those of skill in the medical arts, such as *via* a blood pressure cuff or arterial line as described herein.

It is contemplated that the aforedescribed method may be used in the treatment of a human receiving a chemotherapeutic agent. By way of example, chemotherapeutic agents associated with at least a risk of the development of systemic hypotension include tumor necrosis factor, interleukin-2 and interleukin-1 alone or in combination with interferons or each other.

In a most preferred aspect of the aforedescribed method, the arginine-free parenteral formulation

comprises the mixture of essential and non-essential amino acids already described herein together in a pharmacologically acceptable excipient. It is contemplated that the therapeutically effective concentration of the arginine analog capable of inhibiting nitric oxide production to be used in conjunction with the method is between about 0.1 mg/kg and about 100 mg/kg. A most preferred and narrowly defined range of arginine analog to be used in the described method is between about 10 mg/kg and about 30 mg/kg. Most preferably, the therapeutically effective concentration of the arginine analog constitutes a dose of about 20 mg/kg.

Those arginine analogs most preferred in conjunction with the herein described methods include N^{ω}-methyl-L-arginine, N^{ω}-amino-L-arginine, or N^{ω}-nitro-L-arginine, or a mixture thereof. Among these, N^{ω}-methyl-L-arginine is most particularly preferred.

In still another aspect of the invention, a method for treating hypotension attendant septic shock is provided. In one embodiment of the method, a therapeutically effective amount of an arginine-free parenteral formulation as described herein is administered to an animal concurrently with or prior to the administration of a therapeutically effective amount of an arginine analog capable of inhibiting nitric oxide synthase, and maintaining the animal on the arginine-free parenteral formulation until a systolic blood pressure of at least about 100 mM Hg is detectable in the animal. By way of example, hypotension attendant septic shock is noted in animals with bacterial endotoxin-related septic shock. While any of a variety of animals may be treated for hypotension attendant septic shock according to the aforedescribed method, it is contemplated that the method may find particular applicability in the treatment of

humans suffering from such a condition. In particularly preferred embodiments of the described method, the arginine analog preferred is N$^{\omega}$-methyl-L-arginine, N$^{\omega}$-amino-L-arginine, N$^{\omega}$-nitro-L-arginine or a mixture thereof. Of these arginine analogs, N$^{\omega}$-methyl-L-arginine or N$^{\omega}$-amino-L-arginine is most preferred.

In still another aspect of the invention, a method for providing nutritional support for an animal with hypotension or at risk of developing hypotension is provided. This method comprises administering to the animal a nutritionally supportive arginine-free parenteral formulation concurrently with or prior to treatment with a hypotension inhibiting concentration of a nitric oxide synthase inhibitor. Subjects at risk of developing hypotension include subjects in shock or having had experienced some sort of trauma, subjects exposed to endotoxic agents or a chemotherapeutic agent having hypotensive activity.

The nitric oxide inhibitors of the present invention may be even more specifically described as a nitric oxide synthase inhibitors.

A TPN regimen of low or essentially arginine-free formulations is proposed in conjunction with a treatment regimen of nitric oxide synthase inhibitors to reduce, if not eliminate, the risk of hypotension and septic shock in patients with bacterial infections. Clinical regimens which typically require the administration of a TPN formulation include, for example, nutritional support of cancer patients and others who have no or limited ability to tolerate oral feeding.

Preferred N$^{\omega}$-substituted arginine analogs of the L configuration for uses as described herein include N$^{\omega}$-aminoarginine, N$^{\omega}$-nitroarginine, and N$^{\omega}$-alkyl arginines

such as N$^{\omega}$-methylarginine, N$^{\omega}$-ethylarginine, N$^{\omega}$-propylarginine or Nω-butylarginine. Therapeutically effective amounts of the substituted or disubstituted arginine analogs inhibit production in the animal or patient of nitric oxide from arginine, thus obviating its hypotensive effects. Notwithstanding the accumulated evidence supporting synthesis of NO·, it is understood by those skilled in the art that other nitrogen oxides may be present and may be active in reducing blood pressure.

The present invention also contemplates the use of additional guanidino-based inhibitors of nitric oxide synthesis for the treatment of hypotension, septic shock, and related conditions, in combination with the arginine-free parenteral formulations of the present invention. These guanidino-based inhibitors comprise molecules that are smaller than L-arginine (molecules containing 5 or fewer carbon atoms). The inventors have found that guanidino containing amino acids that include a carbon chain of one less carbon than arginine do not function as nitric oxide synthase inhibitors, while guanidino containing amino acids that have a carbon chain length one carbon longer than arginine have some albeit reduced, nitric oxide synthase inhibiting activity. Guanidino containing amino acids which include a carbon chain two carbons longer than L-arginine were found not to have nitric oxide inhibiting activity. The present inventors have also found that arginine analogs that may inhibit nitric oxide synthesis (or nitric oxide synthase) do not require a carboxyl group or one of the amino groups of the native arginine molecule to retain nitric oxide synthase inhibiting activity. Therefore, the present invention is intended to encompass nitric oxide synthase inhibitors and arginine analogs that include these guanidino-based compounds. Examples of these guanidino-based compounds include amino guanidine and N$^{\alpha}$-alkyl-N$^{\alpha'}$-

amino guanidines where the alkyl group contains 1 to 3 carbons.

The inventors herein also propose uses of anti-TNF antibodies, anti-endotoxin antibodies, and cytokine receptor antagonists (e.g., IL-1 receptor antagonist) in combination with arginine-free formulations in conceptually distinct approaches for the treatment of septic and cytokine-induced shock. The arginine-free formulations may be conveniently supplied as either a parenteral formulation (to be administered intravenously) or as an oral formulation (to be administered enterally). The most preferred embodiment of the parenteral formulation comprises a parenteral formula.

Since antibodies and receptor antagonists intervene early in the pathological response to cytokines, their optimal use requires anticipation of shock. While administration of these agents could affect important biological events after the initiation of the biological response to endotoxin or cytokines, these agents do not block NO synthesis once the enzyme is induced. Because NO production, once induced in smooth muscle cells, appears to persist for more than 16 hours after transfer to cytokine-free medium, antibodies and receptor antagonists are less likely to be effective at blocking or slowing NO synthesis once it has begun. The recently reported potential benefit of a human monoclonal antibody against endotoxin in patients with septic shock associated with gram-negative bacteremia (Ziegler *et al.* (1991) *New England Journal of Medicine, 324*:429) is best attributed to the prevention of further induction of cytokine mediators rather than to direct improvement of the existing cardiovascular profile. In contrast, the inventors own work has demonstrated that nitric oxide synthase inhibitors or treatments designed to act to

limit nitric oxide synthase substrate availability, act within minutes, even in severe shock.

The rapidity of the response to nitric oxide synthase inhibitors and to treatments designed to limit nitric oxide synthase substrate availability, and the fact that their efficacy is independent of the precipitating factor (i.e., endotoxin, TNF, IL-1 or IL-2) are important clinical considerations that the present inventors have focused in the combination therapies and regimens of the present invention.

According to one aspect of the present invention, a therapeutic regimen comprising a therapeutically effective amount of an arginine-free formulation to be administered concurrently with or followed by a therapeutically effective amount of an anti-endotoxin antibody is provided. The formulation is further defined as comprising a mixture of essential and nonessential amino acids together in a pharmacologically acceptable excipient while the formulation may be prepared as either a parenteral or enteral formulation; a parenteral formulation is most preferred. The anti-endotoxin antibody may comprise either a polyclonal antibody or a monoclonal antibody. Most preferably, however, the anti-endotoxin antibody is a monoclonal antibody. By way of example, such a monoclonal antibody is HA-1A.

The parenteral formulation of the therapeutic regimen is described *infra*, again constituting a mixture of essential and non-essential amino acids.

In a most preferred embodiment of the described therapeutic regimen, the parenteral formulation is to be administered concurrently (simultaneously) with the administration of the anti-endotoxin antibody. The therapeutically effective amount of the formulation is

most particularly defined as an amount sufficient to reduce plasma or serum concentrations of arginine in the animal. A reduction in plasma or serum concentrations of arginine in the animal is anticipated to be accomplishable by administering to the animal a continuous intravenous feed of the arginine-free formulation as a parenteral intravenous feed for at least 2 hours. It is anticipated that a preferred practice of using the therapeutic regimen will include the administration of the arginine-free formulation concurrently with the administration of the anti-endotoxin antibody. Simultaneous or concurrent administration is preferred primarily due to the need for the various treatments to act so as to provide the most rapid control of the hypotensive condition.

While the anti-endotoxin antibody may be administered to the animal *via* a number of routes known to those of skill in the medical arts, intravenous administration of the anti-endotoxin antibody is most preferred.

In still another aspect of the present invention, a therapeutic regimen comprising a therapeutically effective amount of an arginine-free formulation administered concurrently with or followed by a therapeutically effective amount of an interleukin-1 receptor antagonist is provided. Again, the parenteral formulation may be described as comprising a mixture of essential and nonessential amino acids together in a pharmacologically acceptable excipient. The parenteral formulation of this therapeutic regimen will include the amino acids and concentrations thereof already recited, and may also include ornithine or citrulline, or both.]

The most preferred route of administration of the interleukin-1 receptor antagonist is *via* intravenous

administration. However, other routes of administration may be used with equal efficacy in the practice of the claims therapeutic regimen. A most particularly preferred interleukin-1 receptor antagonist to be used in the present invention is IL 1ra. Most preferably, the arginine-free parenteral formulation is to be administered concurrently with the interleukin-1 receptor antagonist.

The present invention also includes a therapeutic regimen which comprises a therapeutically effective amount of an arginine-free formulation to be administered concurrently with or followed by a therapeutically effective amount of an anti-tumor necrosis factor antibody. Again, the arginine-free formulation is to comprise a mixture of essential and nonessential amino acids together in a pharmacologically acceptable excipient, and is most preferably to be administered concurrently with the anti-tumor necrosis factor antibody. While the formulation may be either a parenteral or enteral formulation, parenteral formulations are most preferred.

The anti-tumor necrosis factor antibody may comprise either a monoclonal antibody or a polyclonal antibody, with the monoclonal antibody for tumor necrosis factor being most preferred. These antibodies may be prepared according to methods well known to those of skill in the art, including standard immunization protocols and/or hybridoma technologies. Tumor necrosis factor may be obtained from commercial sources for such methods. For example, tumor necrosis factor may be obtained from Amgen Biologicals (Thousand Oaks, California). By way of example, an anti-tumor necrosis factor monoclonal antibody which may be used in the practice of the invention is CB0006, which is described in Exley *et al.* (1990) Lancet, 335:1275-1277. The Exley *et al.* reference

is specifically incorporated herein by reference for this purpose.

It is contemplated that the therapeutically effective amount of the anti-tumor necrosis factor antibody to be used in the described therapeutic regimen is about 0.1 mg/kg to about 20 mg/kg. Most preferably, it is anticipated that a therapeutically effective amount of anti-tumor necrosis factor will be provided to the animal at a dose of about 10 mg/kg. The most preferred route of administration of the anti-tumor necrosis factor antibody is *via* intravenous administration. Most preferably, the parenteral formulation is to be administered concurrently with the administration of the anti-tumor necrosis factor antibody, and in an amount sufficient to reduce plasma or serum concentrations of arginine.

It is contemplated that the amount of arginine-free parenteral formulation that will be sufficient to reduce plasma or serum concentrations of arginine in the animal constitutes an intravenous feed of the arginine-free parenteral formulation as described herein for at least 2 hours. In a preferred aspect of the described therapeutic regimen, the arginine-free parenteral formulation is to be administered concurrently with the administration of the anti-tumor necrosis factor antibody.

In still another aspect of the described invention, a method for treating hypotension attendant to septic shock is provided. The method comprises administering to the animal a therapeutically effective amount of an arginine-free formulation concurrently with or prior to administering a therapeutically effective amount of an anti-endotoxin antibody, periodically monitoring blood pressure in the animal until a systolic blood pressure of

at least 100 mm Hg is detectable in the animal, and maintaining the animal on the arginine-free parenteral formulation until a systolic blood pressure of at least 100 mm Hg for at least 24 hours is established. This method is anticipated to be most particularly preferred in the treatment of hypotension attendant that septic shock which is a bacterial endotoxin-related septic shock. The arginine-free formulation is most preferably prepared as a parenteral formulation.

The anti-endotoxin antibody of the described method may be either a monoclonal antibody or a polyclonal antibody, with monoclonal antibodies to endotoxin being most particularly preferred. By way of example, such an anti-endotoxin monoclonal antibody is designated HA-1A, which is described in detail in the examples included herein. A therapeutically effective concentration of the anti-endotoxin antibody as part of the herein-described method is defined as constituting a concentration of between about 0.1 mg/kg to about 20 mg/kg.

Another aspect of the method comprises a method for treating chemotherapeutic agent-related hypotension. In a most preferred embodiment, the method comprises monitoring an animal receiving a chemotherapeutic agent for a decrease in systolic blood pressure to less than about 100 mm Hg to detect an animal with systemic hypotension, treating the animal having systemic hypotension with a therapeutic regimen comprising a therapeutically effective amount of an arginine-free formulation sufficient to reduce plasma or serum arginine concentrations administered concurrently with or followed by the administration of a therapeutically effective concentration of an interleukin-1 receptor antagonist or an anti-tumor necrosis factor antibody, and maintaining the animal on the therapeutic regimen until an increase of systolic blood pressure to at least about 100 mm Hg is

detectable. Most preferably, the arginine-free formulation is a parenteral formulation. The formulation is most preferably administered concurrently with an antagonist or antibody.

It is contemplated that the described method may be useful in the treatment of a human receiving a chemotherapeutic therapeutic agent associated with the development of hypotension. By way of example, chemotherapeutic agents associated with a decrease in blood pressure, i.e. hypotension, include tumor necrosis factor, interleukin-1 and interleukin-2.

Where the chemotherapeutic agent being administered to the animal is tumor necrosis factor, the therapeutic regimen will include the administration of an interleukin-1 receptor antagonist, such as IL 1ra. In contrast, where the chemotherapeutic agent being used is interleukin-1 or interleukin-2, the therapeutic regimen will include an anti-TNF antibody.

Ordinarily, patients receiving TNF would not preferentially receive anti-TNF antibodies. However, if severe refractory hypotension occurs, then anti-TNF antibody treatment combined with an arginine-free TPN may be useful even where TNF is the chemotherapeutic agent being used. Such constitute potential rescue strategies in treating patients with refractory hypotensive conditions.

Again, the arginine-free parenteral formulation to be used in conjunction with either the interleukin-1 antagonist or anti-tumor necrosis factor antibody includes the amino acids and concentrations thereof already described herein, together in a pharmacologically acceptable excipient. The formulation may also further include ornithine, citrulline, or both, for the reasons

described herein. The therapeutically effective concentration of the interleukin-1 receptor antagonist is more particularly defined as constituting a concentration of between about 1 mg/kg to about 100 mg/kg. The anti-tumor necrosis factor antibody most preferred for use in the described method is a monoclonal antibody, and will constitute a therapeutically effective concentration thereof of between about 0.1 mg/kg to about 20 mg/kg.

The present invention also provides a method for treating septic shock-related hypotension in an animal exposed to endotoxin. This method, in one preferred embodiment, comprises treating the animal with a therapeutically effective amount of an arginine-free formulation sufficient to reduce plasma or serum arginine levels in the animal concurrently with an interleukin-1 receptor antagonist or an anti-endotoxin antibody, or both, monitoring the blood pressure of the animal, and maintaining the animal on the arginine-free parenteral formulation until a systolic blood pressure of at least 100 mm Hg is detected. Most preferably, the formulation is a parenteral formulation.

The present invention also provides a method for treating hypotension in an animal with endotoxic related septic shock or cytokine-induced shock. The method comprises administering to an animal a therapeutically effective amount of an arginine-free formulation sufficient to reduce plasma or serum arginine levels in the animal, administering the formulation concurrently with or prior to the administration of a therapeutically effective amount of an anti-tumor necrosis factor antibody, monitoring blood pressure of the animal, and maintaining the animal on the arginine-free formulation until a systolic blood pressure of at least 100 mm Hg is detected. While the anti-tumor necrosis factor antibody may be a monoclonal or a polyclonal antibody, monoclonal

antibodies are most preferred. Again, the arginine-free formulation is most preferably a parenteral formulation.

The arginine-free parenteral formulation of the method includes the amino acids and amounts thereof previously described herein together in a pharmaceutically acceptable excipient. Again, the formulation may include ornithine, citrulline, or both. It is contemplated that the therapeutically effective amount of the anti-tumor necrosis factor antibody to be used in conjunction with the described method constitutes an amount of between about 0.1 mg/kg to about 20 mg/kg body weight of the animal. Of this range, a dose of about 10 mg/kg of the anti-tumor necrosis factor may constitute the most effective anti-hypotensive dose when used in conjunction with the arginine-free parenteral formulation. So implemented, it is anticipated that the herein described methods may be useful in the treatment of endotoxin-related septic shock or cytokine-induced shock in a human.

While the anti-tumor necrosis factor antibody or interleukin-1 receptor antagonist may be administered to the animal *via* any route known to those of skill in the medical arts, it is most preferred that these agents be administered intravenously, most preferably as a separate bolus dose to the animal. The aforedescribed method is anticipated to provide a most preferred embodiment for treating cytokine-induced shock in an animal.

Notwithstanding the accumulated evidence supporting synthesis of NO, it is understood by those skilled in the art that other nitrogen oxides may be present and may be active in reducing blood pressure. Within this specification, the acronym NO· will be understood to represent nitric oxide and any additional vasoactive nitrogen oxides.

Abbreviations used in the drawings and other places in the present disclosure include the following.

ACh = acetylcholine
BAEC = bovine aortic endothelial cells
B.P. = blood pressure
CO = Cardiac output
EDRF = Endothelium-Derived Relaxing Factor
ET = endotoxin
GP = guinea pig
HIST = histamine
IFN = gamma-interferon
IV = Intravenous
L-Arg = L-arginine
LPS = lipopolysaccharide (endotoxin)
L-NMA = L-NMMA = N^w-methyl-L-arginine
MAP = mean arterial pressure
MBEC = murine brain endothelial cells
NAA = N^w-L-amino-L-arginine
NNA = N^w-nitro L-arginine
NO = Nitric Oxide
PAF = Platelet Activating Factor
PPS = Platelet - poor, plasma-derived serum
SAP = Systemic arterial pressure
SNP = sodium nitroprusside
SVR = Systemic vascular resistance
TNF = Tumor Necrosis Factor
TPN = Total Parenteral Formulation

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 - Reversal of hypotension associated with the administration of IL-1 to dogs. The tracing shown is a representative experiment that was repeated four times. Each dog was anesthetized and incubated as described herein, and baseline cardiovascular data were monitored for 30 minutes. IL-1 was dissolved in phosphate-buffered

saline (pH 7.4) containing 1 mg/ml of dog albumin, and the resulting solution was infused intravenously for three minutes to deliver a dose of 50 μg/kg. MAP was continuously monitored; when a stable nadir was reached and maintained for more than 10 minutes or when MAP dropped below 50 mm Hg, NAA (20 mg/kg in 10 ml phosphate-buffered saline) was administered by intravenous infusion for 30 seconds. Sixty minutes after MAP was restored to baseline or higher values, L-arginine (400 mg/kg in 20 ml phosphate-buffered saline) was administered by slow intravenous infusion for 5 minutes. Anesthetized dogs given IL-1 (50 μg/kg) developed a progressive, moderately severe hypotension, with MAP decreasing from a baseline of 111 +/- 7 mm Hg to 80 +/- 2 mm Hg within about 3 hours. Hypotension was rapidly reversed by the intravenous administration of a single bolus dose of NAA (20 mg/kg, 88 umol/kg), with MAP increasing by from 46 +/- 9 mm Hg (58%_ to 125 +/- 8 mm Hg.

FIGURES 2A, 2B, 2C - Inhibition of IL-1-activated NO· production by N$^{\omega}$-substituted arginine analogues. Figure 2A: Comparison of nitrite synthesis inhibition in smooth muscle cells by the L-isomers of NAA, NMA, and NNA and by the D-isomer of NMA. Figure 2B: Concentration dependence of nitrite synthesis inhibition in smooth muscle cells by NAA as a function of different L-arginine concentration in the culture medium. Confluent monolayers of smooth muscle cells in 96-well microtiter plates were treated with a combination of 100 ng/ml IL-1 and 50 ng/ml IFN-γ for 24 hours. Cells were then washed with Hanks' balanced salt solution, and fresh medium containing the indicated concentration of L-arginine was added. Points indicate means ± SD of nitrite production, expressed as percent of control, during a 16-hour exposure to arginine analogues (n = 3-4). Figure 2C: Effect of NAA on smooth muscle cell NO· synthase. Cytosol from cytokine-induced smooth muscle cells (~2 μg

protein) was incubated with 1 m*M* L-arginine, 0.5 m*M* NADPH, 1 m*M* dithiothreitol, 10 μM flavin-adenine dinucleotide, 10 μM tetrahydrobiopterin, and 0.1 U/ml dihydropteridine reductase in a total volume of 100 μL. The time course of Fe^{2+}-myoglobin oxidation was measured in the absence (control) or presence of 300 μM NAA (+NAA). The inventors have also observed that myoglobin oxidation showed an absolute dependence on L-arginine and NADPH. Points indicate the time course of change in rate of NO· synthesis in a representative experiment which was repeated three times.

FIGURE 3 - Illustrates the effects of the sequential administration of NMMA and L-arginine on moderately low blood pressure of a dog treated with TNF.

FIGURE 4A - Illustrates the effects of sequential administration of NMMA and L-arginine on severe hypotension in a canine administered TNF.

FIGURE 4B - Illustrates control experiments where NMMA was administered to previously untreated dogs.

FIGURE 4C - Illustrates the effects of NMMA on nitroglycerin-induced canine hypotension.

FIGURE 5 - Shows the time course of changes in mean systemic arterial pressure (SAP) in a pentobarbital-anesthetized dog following the IV administration of endotoxin (LPS; Lipopolysaccharide) (ET), N^{ω}-methyl-L-arginine (L-NMA), and L-arginine (L-Arg).

FIGURE 6 - Shows the time course of changes in mean systemic arterial pressure (SAP) in a pentobarbital-anesthetized dog following the i.v. administration of endotoxin, L-NMA (2 doses) and L-Arg.

FIGURE 7 - Shows the time course of TNF-mediated canine systemic hypotension and reversal by Nω-aminoarginine.

FIGURE 8 - Shows the reversal of endotoxin-induced systemic hypotension by N$^{\omega}$-aminoarginine (3 doses).

FIGURE 9A-B - IL-1 activates nitrite production by rat aortic smooth muscle cells in culture. **FIGURE A:** Time course of nitrite synthesis elicited by 40 ng/mL IL-1 alone (open circles) and by IL-1 in the presence of 50 ng/ml IFN-γ (open triangles). In the absence of IL-1, IFN-γ did not elicit nitrite production. Inclusion of IL-1 receptor antagonist (40 μg/mL; filled symbols) in the culture medium inhibited nitrite production stimulated by both IL-1 and IL-1 plus IFN-γ. **FIGURE B:** IL-1 concentration dependence for smooth muscle cell nitrite production. Data points indicate nitrite produced during a 29-hour incubation with the indicated concentration of IL-1 alone (open circles) or in the presence of IL-1 plus 50 ng/mL IFN-γ (open triangles). All values are means ± SD for nitrite production by confluent smooth muscle cell monolayers grown in 96-well microtiter plates (60-80 X 10^3 cells per well; n = 3-4).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Clinical studies of biologic response modifiers such as certain cytokines have shown that a major dose-limiting toxicity associated with administration of such agents is hypotension. Nitrite, the predominant spontaneous oxidation product of NO, is readily assayed and used herein for assays of NO production. Nitric oxide (NO) is a highly reactive compound which spontaneously decomposes to nitrate and nitrite in a culture medium.

The present invention involves implements the inventors' findings that IFN (100 U/ml) in combination with either TNF (500 U/ml), IL-1 (10 U/ml), or endotoxin (1 μg/ml.), can induce MBEC's to accumulate nitrite in a culture medium (15 to 80 μM) within 48 hours. TNF, IL-1 or endotoxin alone was found to induce the production of minimal levels of nitrites (1-3 μM).

The release of vasoactive factors such as NO by endothelial cells may play a role in the development of hypotension associated with the administration of these agents *in vivo*. This invention relates to a demonstration that cultured MBEC's produce NO in response to various combinations of cytokines and the potential role of NO in the pathophysiological causes of hypotension and cardiovascular collapse.

The present inventors have found that increased concentrations of nitrites were not associated with MBEC exposed to TNF and IFN in arginine-free culture medium. The nitrite concentration was found to increase in a dose dependent manner upon addition of L-arginine back to the medium of a culture of MBEC cells. The inventors have also found that nitrite accumulates when cultured mouse endothelial cells are exposed to immunomodulators and endotoxin[17].

The present invention relates to a method for inhibiting or preventing hypotension as well as septic shock through the reduction of nitric oxide synthesis. Most particularly, the method employs the administration of an arginine-free parenteral formulation to effect a reduction in serum arginine levels. This reduction in serum arginine levels is postulated to provide for a reduction in nitric oxide synthesis, as arginine is the substrate from which nitric oxide is formed. Nitric

oxide has recently been demonstrated by the inventors to be derived from the amino acid L-arginine.

As nitric oxide is a potent vasodilator linked to the development of hypotension and septic shock, the claimed methods propose the inhibition and prophylaxis of these conditions in an animal through the control of serum arginine concentrations and nitric oxide synthesis. The invention provides a reduction in nitric oxide synthesis by eliminating or limiting the arginine concentration in an animal's serum. A reduction in an animal's serum concentration of arginine is accomplished through restricting the amount of arginine given to the animal to less than about 0.1% arginine in a non-hypotensive TPN formulation.

Particular embodiments of the claimed formulations may include ornithine and citrulline, ornithine alone, or citrulline alone to supplement urea cycle substrate physiological requirements in the animal.

For purposes of the present invention, the term "cytostatic" is defined as that physiological state of a cell characterized by a lack of active cell division. Thus, a culture or group of cells which are in a "cytostatic" state are not actively dividing or which are growth inhibited (i.e., virtually no cell growth).

The terms "anti-hypotensive" and "non-hypotensive" are used interchangeably in defining the present invention. These terms are intended to denote the nature of the formulation as limiting the onset of hypotension through decreased arginine concentrations and thus availability in the serum of arginine.

For purposes of the present invention, a "pressor" agent is defined as a pharmacological agent which causes

an increase in blood pressure when administered to an animal. By way of example, phenylephrine is a "pressor" agent. However, other pressor agents would be expected to provide similar effects in the systems described herein. Other examples of pressor agents include dopamine, epinephrine, norepinephrine and phenylephrine.

The present invention also provides for unique therapeutically useful regimens for treating hypotension, such as that attendant septic shock or treatment with certain chemotherapeutic agents such as TNF, IL-1 or IL-2. The various regimens and formulations thereof described in conjunction with the present invention include the administration of an arginine-free parenteral formulation. The formulation is to be administered either concurrently with or followed by the treatment of the animal with an arginine analog capable of inhibiting nitric oxide, production. Agents which are capable of inhibiting nitric oxide production include, arginine analogs capable of inhibiting nitric oxide synthase, and include NMA, NAA, NNA, or a mixture thereof.

Human recombinant IL-1-α (hereafter referred to as IL-1; specific activity, 2×10^7 lymphocyte-activating factor units/mg) was produced by Dainippon Pharmaceutical Co. LTD. and provided by the National Cancer Institute. Human recombinant IL-1 receptor antagonist was produced by Synergen, Co. and was provided by Drs. Lyle Moldawer and Stephen Lowry, Department of Surgery, Cornell University Medical College. Rat interferon-γ (IFN-γ) was obtained from Amgen Biologicals (Thousand Oaks, CA).

N^{ω}-methyl-L-arginine (NMA) and NAA, hydrochloride salt, were synthesized as reported previously by Gross *et al.* (1990) (*Biochem. Biophys. Res. Commun., 170*:96-103) and Corbin (1974) (*Anal. Biochem., 57*:310-312), which references are specifically incorporated herein by

reference for this purpose. Except where indicated, all biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture media and reagents, unless otherwise noted, were from Whittaker Bioproducts (Walkersville, MD).

Cell Culture

Mouse A375 melanoma cells were provided by Dr. E. Kleinerman, The University of Texas M.D. Anderson Cancer Center. Cells were maintained in Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1) containing 10 m*M* HEPES buffer (pH 7.4) and 10% fetal bovine serum. All tissue culture reagents contained less than 0.25 ng/ml endotoxin as measured by the limulus amebocyte assay. Murine D10 T cells were obtained from the American Type Culture Collection (Rockville, MD).

Aortic smooth muscle cells were cultured by explanting segments of the medial layer of aortas from adult male Fischer 344 rats. Aortas were removed aseptically and freed of adventitial and endothelial cells by scraping both the luminal and abluminal surfaces. Medial fragments were allowed to attach to Primaria 25-cm^2 tissue culture flasks (Becton-Dickinson, Lincoln Park, NJ) which were kept moist with growth medium until cells emerged. Cultures were fed twice weekly with medium 199 containing 10% fetal bovine serum, 25 m*M* HEPES buffer (pH 7.4), 2 m*M* L-glutamine, 40 μg/ml endothelial cell growth supplement (Biomedical Technologies, Inc. (Stoughton, MA), and 10 μg/ml gentamicin (GIBCO). When primary cultures became confluent, they were passaged by trypsinization, and explants were discarded. For these studies, cells from passages 12-14 were seeded at 20,000/well in 96-well plates and were used at confluence (60,000-80,000 cells/well). The cells exhibited the classic SMC

phenotype with hill and valley morphology and stained positively for smooth-muscle actin.

Cell Respiration Assay

Rat aortic smooth muscle cells in 96-well microtiter plates were incubated for 90 minutes in RPMI-1640 medium containing 0.2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), washed with Hands' balanced salt solution, and solubilized in 100 µl of dimethyl sulfoxide. The extent of reduction of MTT to formazan within cells, quantitated by measurement of the optical density at 550 nm (OD_{550}), was taken as an indicator of cellular respiration. The assay is more thoroughly described by Klostergaard *et al.* (1987) (*J. Immunol. Methods, 101*:97-108), which reference is specifically incorporated herein by reference for this purpose.

IL-1-Induced Cell Proliferation Assay

Murine D10 cells, an IL-1-dependent T-cell line, were used to measure IL-1 mitogenic activity. Cell proliferation in the presence of IL-1 was assessed by incorporation of [^{3}H]thymidine as previously described by Bakouche *et al.* (1987) (*J. Immunol., 138*: 4249-4255), which reference is specifically incorporated herein by reference for this purpose.

IL-1-Induced Cytotoxicity Assay

IL-1-induced cytotoxicity was studied using A375 tumor cells plated at a density of 6000 cells per well in 96-well microtiter plates. After overnight attachment, IL-1 (3-300 ng/ml) was added in the presence or absence of NAA or NMA. After cells were incubated for 3 days, [^{3}H]thymidine was added (1 µCi per well) for an additional 2 hours. Cells were harvested onto glass fiber disks (PHD Cell Harvester; Cambridge Technology, Inc., Watertown, MA). Disks were air dried overnight, and

radioactivity was determined with a Model 1900TR Scintillation Counter (Packard Instrument Division, Downers Grove, IL).

Induction and Assay of Nitrite Synthesis in Smooth Muscle Cells

Rat aortic smooth muscle cells were incubated with RPMI-1640 medium containing 10% bovine calf serum, 25 m*M* HEPES buffer (pH 7.4), 2 m*M* glutamine, 80 U/ml: fungizone, and IL-1, IFN-γ, and various inhibitors at the concentrations indicated in the figure legends. At the desired times, nitrite concentration in the culture medium was measured using the standard Griess assay adapted to a 96-well microtiter plate reader.[36] The Griess assay is also described in Gross *et al.*[37] (1991), which reference is specifically incorporated herein by reference for this purpose. Thus, 100 μL of Griess reagent (0.5% sulfanilic acid, 0.05% naphthalenediamine, and 2.5% phosphoric acid) was added to an equal volume of culture medium, and the OD_{550} was measured and related to nitrite concentration by reference to a standard curve. The background OD_{550} of medium incubated in the absence of cells was subtracted from experimental values.

Preparation and Assay of Smooth Muscle Cell NO· Synthase

Rat aortic smooth muscle cells were incubated with RPMI-1640 medium containing 10% bovine calf serum, 25 m*M* HEPES buffer (pH 7.4), 2 m*M* glutamine, 80 μg/ml penicillin, 80 μg/ml streptomycin, 2 μg/ml fungizone, 30 μg/ml lipopolysaccharide (*Escherichia coli* 0111:B4), and 50 U/ml IFN-γ. Cells were harvested after 24 hours, and cytosol was prepared as described in the above cited Gross *et al.* (1991)[41] article. Cytosolic NO· synthase activity was assayed by the Fe^{2+}-myoglobin method described previously.

Studies of IL-1-Induced Hypotension in Dogs

Experiments were conducted on healthy mongrel dogs that were free of microfilaria and weighed 25-28 kg each. All protocols were approved by The University of Texas Animal Welfare Committee, and animal care met all standards prescribed in the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education and Welfare, Guide for the Care and "Use of Laboratory Animals Pub. No. 78-23, Washington, DC, Health Department (1978). After an overnight fast, the dogs were anesthetized with pentobarbital (25 mg/kg given intravenously), orotracheally incubated, and ventilated with an animal respirator (Harvard Apparatus, South Natick, MA), using room air at a tidal volume of 15 ml/kg delivered at 14 breaths per minute and adjusted to normal pH and CO_2 tension. A catheter was placed percutaneously in the femoral artery, and a flow-directed thermodilution catheter was inserted through the jugular vein and positioned with the distal port in the pulmonary artery. Mean systemic arterial pressure (MAP), heart rate, cardiac output, and systemic vascular resistance were measured as previously described in Kilbourn *et al.* (1990) (*Biochem. Biophys. Res. Commun., 172*:1132-1138), which reference is specifically incorporated herein by reference.

The therapeutic regimens and methods described herein by the inventors provide a novel and potentially more effective method for treating a variety of conditions where hypotension is a typical consequence. For example, hypotension is typically seen in patients suffering from septic shock and in those receiving certain chemotherapeutic agents (TNF, interleukin-1, interleukin-2). The innovative combination of an arginine-free parenteral formulation together with anti-endotoxin antibodies, anti-tumor necrosis factor antibodies, and/or interleukin-1 receptor antagonist may allow for the use of chemotherapeutic agents without the

risk of the development of life-threatening hypotension in the animal.

Recombinant IL-1 or TNF may be obtained from Amgen (Thousand Oaks, California) and used in standard immunization techniques to provide polyclonal antibodies to these antigens. Alternatively, spleen or other tissue from the immunized animals may be used to prepare hybridoma cell lines when fused with an immortal cell line. Such hybridomes constitute a source of monoclonal antibodies specific for their respective antigens.

For purposes of describing the present therapeutic regimens and methods for the treatment of hypotension, such as in a patient or animal with systemic sepsis, the following symptoms will be monitored: fever or hypothermia (temperature > 38.3°C [101°F] or <35.6°C [96°F]; tachycardia (>90 beats per minute in the absence of beta-blockade) and tachypnea (respiratory rate >20 breaths per minute or the requirement of mechanical ventilation); and either hypotension (systolic blood pressure ≤90 mm Hg or a sustained drop in systolic pressure ≥40 mm Hg in the presence of an adequate fluid challenge and the absence of antihypertensive agents) or two of the following six signs of systemic toxicity or peripheral hypoperfusion: unexplained metabolic acidosis (pH ≤7.3 base deficit of >5 mmol per liter, or an elevated plasma lactate level); arterial hypoxemia (partial pressure of oxygen ≤75 mm Hg or ratio of the partial pressure of oxygen to the fraction of inspired oxygen <250); acute renal failure (urinary output of less than 0.5 ml per kilogram of body weight per hour); elevated prothrombin or partial-thromboplastin time or reduction of the platelet count to less than half the base-line value or less than 100,000 platelets power cubic millimeter; sudden decrease in mental acuity; and cardiac index of more than 4 liters per minute per square

meter of body-surface area with systemic vascular resistance of less than 800 dyn sec cm^{-5}.

Even though the present invention has been described with a certain degree of particularity, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the following disclosure. Accordingly, it is intended that all such alternatives, modifications, and variations which fall within the spirit and the scope of the invention be embraced by the defined claims.

The following examples are presented to describe preferred embodiments and utilities of the present invention, but should not be construed as limiting the claims thereof unless specifically indicated to do so.

EXAMPLE 1

Correlation Between Serum Arginine Levels and Blood Pressure

The present example is provided to demonstrate the correlation between plasma arginine levels and blood pressure. More specifically, the present example demonstrates a correlation between low plasma arginine levels and increased blood pressure in endotoxin-treated animals.

The present example also demonstrates the utility for employing a parenteral formulation which is essentially arginine free or low enough in arginine to lower plasma arginine levels, to prevent or alleviate synthesis of nitric oxide, which in turn is shown to result in an increase in blood pressure. Maintenance of an animal on an arginine-free parenteral formulation and a nitric oxide synthase inhibitor, such as the arginine

analogs NMA, NAA or NNA or a combination thereof, would therefore, according to the present invention, provide a method for even further augmenting the anti-hypotensive effect of both components of such a regimen, using significantly lower doses of arginine analogs than would otherwise be required in animals with normal or high physiological concentrations of arginine. The hypertensive effect of NAA and other nitric oxide inhibitors *in vivo* alone is also described in the present disclosure. The onset of life-threatening levels of low blood pressure, such as that typically attendant to cytokine-induced hypotension and septic shock would thus be prevented or alleviated in an animal. The onset of life-threatening levels of low blood pressure, such as that typically attendant to hypotension and septic shock would thus be prevented or alleviated in an animal.

The enzyme arginase was used to reduce plasma arginine levels in Spraque-Dawly rats (Weight per rat = 250-300 gm). Arginase is an enzyme that irreversibly converts L-arginine to L-ornithine + urea. The rats were anesthetized with ethyl ether and then pithed as described Shiply and Tilden[30]. The animals were pithed prior to use in the present study so as to eliminate any neurological control of blood pressure.

Arginase was dissolved in sterile saline (1000 I.U./ml) and was administered by intravenous infusion at a rate of 300 I.U./min. for 20 min. One I.U. is the amount of arginase that converts 1 μmol of arginine to products per minute. Blood pressure was determined using a pressure transducer connected to an indwelling catheter placed in the carotid artery as described.[12]

Serum arginine concentrations

The administration of arginase to pithed rats with or without exposure to endotoxin (15 mg/kg dose, ip), according to the dose outlined above, resulted in a decrease in plasma arginine levels of from 150 μM to ≤ 4 μM within a few minutes. Plasma arginine remained at levels ≤ 4 μM for at least 1 hour after the arginase infusion was stopped.

Blood pressure recording in the pithed rat.

To record blood pressure, a tracheotomy was first performed on each rat, after which the rats were artificially respired with room air. The left common carotid artery was then cannulated in each rat for blood pressure measurement *via* a Statham pressure transducer (Hato Rey, Puerto Rico) and displayed on a physiogram (Grass Instruments, Qunicy MA). Heart rate was measured from the lead III electrocardiogram.

Two separate groups of animals were examined. The first group of animals, designated the "control" group, received no endotoxin. The blood pressure of the "control" group animals was measured at two different times, once before the administration of arginase and once after the administration of arginase.

The second group of animals, designated the endotoxin group, received a single dose of endotoxin of 15 mg/kg body weight, which was administered at least 6 hours prior to any subsequent arginase treatment. The blood pressure of all animals in both treatment groups was then measured at two different times, again once before arginase treatment and once after arginase treatment.

The results from this example are presented in Table 1.

TABLE 1
EFFECT OF REDUCED PLASMA ARGINASE ON BLOOD PRESSURE

	No Arginase		Arginase	
Control Rats	B.P. (mm Hg)	Average B.P.	B.P. (mm Hg)	Average
1	61	59.8 ± 1.3	61	63.25
2	60		60	
3	60		64	
4	58		68	
	No Arginase		**Arginase**	
Endotoxin Rats (15 mg/Kg)	B.P. (mm Hg)	Average B.P.	B.P. (mm Hg)	Average
1	36	33.2 ± 3.3	44	38.4
2	34		40	
3	28		28	
4	32		36	
5	36		44	

Blood pressure readings for 4 control pithed rats were 61, 60, 60, and 58 mm Hg (average 59.8 ± 1.3 mm Hg) (See Table 1). Following administration of arginase, blood pressure was unchanged in two rats and increased by 4 and 10 mm Hg in 2 other rats (average increase 3.5 ± 4.7 mm Hg, not statistically significant).

Blood pressure readings for 5 rats at 6 hours after giving 15 mg/kg lipopolysaccharide (endotoxin) by intravenous injection was 36, 34, 28, 32, and 36 mm Hg (average 33.2 ± 3.3 mm Hg, See Table 1). Note that the endotoxin-treated rats were clearly hypotensive relative to the controls.

Following administration of arginase, blood pressure in the endotoxin-treated rats increased by 8, 6, 0, 4, and 8 mm Hg (average increase 5.2 ± 3.3 mm Hg). The average blood pressure increase following arginase treatment of the endotoxic, pithed rats was 15.7% (statistically significant, $p < 0.05$).

Overall, this study shows that reducing plasma arginine levels has no significant effect on blood pressure in control animals, but did have a significant effect on blood pressure readings in endotoxic animals. The lack of a demonstrated effect in control animals may be due to the slow rate of NO formation in control animals, so as to negate any requirement for exogenous (i.e. plasma) arginine. Thus, a reduction in plasma arginine levels in such animals would not be a limiting factor for generating NO.

In contrast, the rate of NO formation in endotoxic animals is much faster than in control (non-endotoxic animals), and results in the development of hypotension. In these endotoxic animals, the cells making NO must obtain extra arginine from the plasma. When plasma arginine is very low in endotoxic animals (i.e. after arginase administration), there is not enough arginine available to sustain a pathologically high rate of NO synthesis by cells associated with blood vessel walls (i.e., endothelial cells). Thus, the concentration of NO is reduced, resulting in a concomitant reduction in the level of blood pressure reduction in the vasculature of

the animal. Thus, depletion of serum arginine levels could be used to effect an increase in blood pressure in hypotensive animals.

Thus, use of arginine-free TPN solutions, or solutions sufficiently low in arginine concentration so as to effect a sufficient reduction in plasma arginine levels adequate to limit nitric oxide synthesis, for example, to about 4 μM arginine or less (i.e., 4 nM/ml serum arginine), are expected to have a beneficial effect comparable to that of arginase administration for preventing or treating hypotension, particularly hypotension in animals in septic shock.

EXAMPLE 2
CORRELATION BETWEEN PLASMA ARGININE LEVELS AND RESPONSE TO PRESSOR AGENTS

The present example is provided to demonstrate the correlation between low plasma arginine levels and increased response to "pressor" agents *in vivo* in endotoxin-treated animals.

Animals were pithed as described in Example 1. Blood pressure measurements were obtained also as described in Example 1. Arginase was also prepared according to the method described in Example 1.

Both left and right jugular veins were cannulated for drug administration; and left jugular was used for bolus administration of phenylephrine and the right jugular vein was used for continuous infusion of arginase. All animals from both groups (Control and Endotoxin) received phenylephrine in sequential doses of 0.3, 1.0, 2.0, or 6.0 ug/Kg.

The results from this example are presented in Table 2.

TABLE 2
EFFECT OF ARGINASE AND ENDOTOXIN ON BLOOD PRESSURE

Phenylephrine dose	Blood Pressure Increase (mm Hg)			
	Without Arginase		With Arginase	
	Raw Data	Ave. ± S.D.	Raw Data	Ave. ± S.D.
Study #1: Control Animals				
0.3 μg/Kg	+16,14,32,9	17.8 ± 5.0	+4,9,20,10	13.3 ± 2.5
1.0 μg/Kg	+40,25,36,24	31.3 ± 4.0	+12,17,40,21	22.5 ± 6.1
2.0 μg/Kg	+64,48,58,34	51.0 ± 6.6	+28,30,66,31	38.8 ± 9.1
6.0 μg/Kg	+84,92,122,74	93.0 ± 10.3	+64,72,120,63	79.8 ± 13.4
Study #2: Endotoxic Animals				
0.3 μg/Kg	+2,0,4,2,3	2.2 ± 0.7	+2,0,4,2,2	2.0 ± 0.6
1.0 μg/Kg	+6,4,9,10,6	7.0 ± 1.1	+8,6,16,9,6	9.0 ± 1.8
2.0 μg/Kg	+12,10,24,12+10	13.6 ± 2.6	+19,14,44,20,20	23.3 ± 5.3
6.0 μg/Kg	+25,26,72,26,20	33.7 ± 9.6	+36,46,78,44,38	48.5 ± 7.6

This example shows the effects of endotoxin and of arginase on mean systolic blood pressure response to phenylephrine in pithed rats. Endotoxin (15 mg/kg body weight) was given by intravenous injection 6 hrs before the experiment began; arginase (300 I.U./Min. for 20 min.) was given intravenously to each rat after the "Without Arginase" measurements were made.

Table 2 shows the maximum increase in blood pressure following the phenylephrine dose indicated for each rat ("Raw Data"); note that the data is in pairs since each rat was tested first without arginase (at 0.3, 1.0, 2.0 and 6.0 ug/Kg phenylephrine, in sequence) and was then retested with phenylephrine in the same dose and sequence

after arginase treatment. Thus, the first line of data in the Table shows that four control rats were each tested with 0.3 μg/Kg of phenylephrine. The first control rat showed a blood pressure of 16 mm Hg without arginase, but only 4 mm Hg after arginase administration ("with arginase"). For the second, third and fourth rats, the blood pressure increments were 14, 32 and 9 before arginase, respectively, and 9, 20 and 10 after arginase, respectively.

The data in Table 2 demonstrates that reducing plasma arginine levels through arginase treatment, enhances the "pressor" agent (such as phenylephrine), response in endotoxic animals, reducing the difference observed between endotoxic and control animal blood pressure increases at the same pressor agent dose. Moreover, endotoxic animals pretreated with arginase demonstrated an enhanced "pressor" effect (a statistically greater increase in blood pressure), compared to the pressor response observed in endotoxic animals receiving no arginase (See Table 2).

Endotoxin decreases an animals ability to present the normal hypertensive response (i.e., increase in blood pressure) to phenylephrine. Thus, compare the "Without Arginase" data of control and endotoxic rats at each dose of phenylephrine (Table 2). This effect occurs because the endotoxic animals are making large amounts of nitric oxide from arginine, and that causes hypotension and blunting of the response to phenylephrine.

Arginase administration improves the hypertensive response to a pressor agent, such as phenylephrine, dose. Smaller differences were observed between control and endotoxic animals given arginase (indicating only a small loss of responsiveness) relative to the larger differences between control and endotoxic animals not

given arginase (indicating a large loss in responsiveness).

For example, at a phenylephrine dose of 6 μg/Kg, in animals not given arginase (i.e., having a higher serum arginine concentration), the pressor response to phenylephrine drops from 93.0 ± 10.3 mm Hg (a pharmacologically useful pressor agent response) in control animals to 33.7 ± 9.6 mm Hg (a poor pressor agent response) in endotoxic animals, a difference in pressor response of 59.3 mm Hg. In contrast, at the same phenylephrine dose in animals given arginase (i.e., decreased serum arginine levels), the pressor response in control and endotoxic animals was 79.8 ± 13.4 and 48.5 ± 7.6, respectively, a difference of only 31.3 mm Hg. Thus, depletion of plasma arginine with arginase very significantly restores the "normal" (hypertensive) response to pressor drugs, such as phenylephrine.

The data herein thus demonstrates that hypotension may be controlled, particularly in endotoxic animals, by manipulating an animal's serum arginine concentration. As serum arginine levels may be controlled in part through an animal's nutrition as they are through arginase administration, the present data provides a mechanism whereby hypotension (low blood pressure) may be corrected by maintaining the animal on a low/anti-hypotensive concentration or essentially arginine-free nutritional regimen. As such, the presently disclosed technique may also be used to prevent the development of hypotension in a patient at risk, either through administration of the TPN formulation alone, or through administration of a combination therapy with a pressor agent or one of the nitric oxide inhibitors, such as NMA, NNA, or a combination, thereof, as described herein.

EXAMPLE 3
Anti-hypotensive
Arginine-free TPN formulation

The present example defines an anti-hypotensive TPN formulation of the present invention.

A sterile, non-pyrogenic, stable solution for parenteral administration to a patient having hypotension or at risk of hypotension or systemic shock, particularly those receiving immunomodulatory agents, is prepared from pure crystalline amino acids, which are dissolved in a glucose solution (5% to 20%) in the following concentrations to provide a 2X concentrate TPN or a ready-to-feed TPN formulation, as indicated:

TABLE 3

Amino Acids	2x concentrate mg/100 ml formulation	Final Concentration (Feeding Formulation) g/l
isoleucine	600-800	3-4
leucine	800-1200	4-6
valine	600-800	3-4
phenylalanine	200-400	1-2
methionine	200-400	1-2
lysine	600-800	3-4
histidine	200-400	1-2
threonine	400-600	2-3
tryptophan	100-300	0.5-1.5
tyrosine	50-150	0.25-0.75
alanine	800-1000	4-5
aspartic acid	400-600	2-3
glycine	800-1000	4-5
proline	600-800	3-4
serine	200-400	1-2

To obtain the preferred TPN formulation concentration suitable as a feeding formulation, a volume of 500 ml of the 2X concentrate (defined in Table 3) is mixed with 500 ml of a dextrose solution, for the production of 1 liter of the feeding formulation (i.e., 500 cc of a 2X concentrate of AA and 500 cc of dextrose solution). Most preferably, the dextrose solution is

supplemented with a physiologically acceptable concentration of vitamins and minerals.

The TPN of the present methods may also include glutamic acid (400-600 mg/100 ml of a 2x conc., or 2-3 g/l in a final concentration) and/or taurine (50-100 mg/100 mls. of a 2-fold concentrate; 0.25-0.5 g/l final concentration).

The solution is then filter sterilized into appropriate containers for intravenous fluids. To prepare for administration, the volume is then brought to the desired feeding solution concentration with an equal volume of sterile glucose solution. The TPN as a ready to feed formulation is then to be kept cool. The solution may then be administered to a patient intravenously (I.V.). The pH of the TPN solution must also be adjusted to a physiologically acceptable pH, between 7.0 and 7.4. The formulation is arginine free.

Example 4
Anti-Hypotensive Formulation with Low Arginine

If a formulation of amino acids for a patient having hypotension, or at risk of developing hypotension or septic shock, is desired which includes arginine, the formulation as outlined in Example 3 may be utilized after supplementation with arginine. Arginine will be added to constitute less than 0.1% final concentration by weight of the formulation (about 1 g. of arginine/liter of the TPN feeding formulation). The same amounts of the essential and non-essential amino acids (leucine, isoleucine, valine, phenylalanine, lysine, valine, isoleucine, threonine, tryptophan, histidine, lyrosine, alanine, glycine, proline and serine) as defined in Example 3 will be present; and the solution prepared in the same manner.

Example 5
Anti-Hypotensive
Arginine-Free TPN Formulation

If a formulation of amino acids for patients at risk of hypotension or septic shock, or receiving pharmacological agents which may cause such a condition, such as TNF (tumor necrosis factor), etc., is desired which is essentially arginine-free and contains ornithine and citrulline, the formula as outlined in Example 3 supplemented with ornithine and citrulline can be utilized.

Ornithine will then be added to constitute between about 1-2 grams ornithine per liter of the TPN feeding solution (between 0.1% and 0.2% by weight ornithine). Citrulline will be added to constitute about 1 gram citrulline per liter of the TPN feeding solution (about 0.1% by weight citrulline). The same amounts of the amino acids of Example 3 will be present, and the solution prepared in the same manner. The anti-hypotensive formulation is arginine free.

PROPHETIC EXAMPLE 6
Formulations and Methods for
Inhibiting Hypotension and Septic Shock

The present prophetic example provides methods whereby the particularly defined arginine-free and low-arginine formulations may be used in the treatment of patients at risk of developing hypotension and septic shock, or whom may require parenteral nutritional support and have already developed hypotension or septic shock.

The proposed formulations and proposed methods may be used most particularly in the clinical management of patients requiring total parenteral nutritional support

and receiving immunomodulators. By way of example, the term "immunomodulator" refers to such agents as interferon, interleukin-2, and tumor necrosis factor.

Many of the class of substances recognized as immunomodulators are used as anti-cancer chemotherapeutic agents. Thus, it is envisioned that the presently described methods would be effective for the clinical management of patients being maintained on parenteral nutritional support and receiving chemotherapeutic agents with immunomodulatory action.

According to the present invention, a method for prophylaxis or treatment of systemic hypotension related to the elevated production of nitric oxide in an animal is provided comprising administering to the animal a non-hypotensive formulation comprising a mixture of amino acids in a pharmaceutically acceptable diluent. The formulation may be essentially arginine-free, in a most preferred embodiment of the method. A non-hypotensive formulation which includes a low arginine concentration may also be employed in another embodiment of the claimed method.

The formu ation is to be administered to the patient until a physiologically acceptable blood pressure in the animal is reached and maintained. For a human, a physiologically acceptable systolic blood pressure level is about 100 mm Hg.

More particularly, the method of the present invention includes an essentially arginine-free or low arginine (between about 0.001% and about 0.1%) formulation comprising a mixture of amino acids. The formulation should be prepared so as to be physiologically suitable as an intravenous

hyperalimentation (total parenteral nutrition) solution for patients requiring such solutions.

Stated as a range of concentrations for the most preferred mixture of amino acids, the formulation of the presently disclosed methods and specially tailored arginine-free formulations is defined in Table 4. Most preferably, the proposed concentrations to be included in such a formulation appear in Table 4.

TABLE 4
PREFERRED RANGES OF AMINO ACIDS IN NON-HYPOTENSIVE FORMULATIONS

about 3-4 g/l isoleucine (0.3-0.4%);
about 4-6 g/l leucine(0.4-0.6%);
about 3-4 g/l lysine (0.3-0.4%);
about 1-2 g/l methionine (0.1-0.2%);
about 1-2 g/l phenylalanine (0.2-0.2%);
about 2-3 g/l threonine (0.2-0.3%);
about 0.5-1.5 g/l tryptophan (0.05-0.15%);
about 3-4 g/l valine (0.3-0.4%);
about 4-5 g/l alanine (0.4-0.5%);
about 1-2 g/l histidine (0.1-0.2%);
about 3-4 g/l proline (0.3-0.4%);
about 1-2 g/l serine (0.1-0.2%);
about 0.25-0.75 g/l tyrosine (0.025-0.075%);
about 4-5 g/l glycine (0.4-0.5%); and
about 2-3 g/l aspartic acid (0.2-0.3%).

The formulation may also include ornithine. Where ornithine is part of the particular formulation, it is to be included at a concentration of about 1-2 grams/l of the TPN feeding formulation (about 0.1-0.2% ornithine).

Where the formulation is a parenteral formulation, the mixture should be adjusted so as to be

physiologically compatible for parenteral administration.

The described non-hypotensive parenteral nutritional formulations may alternatively include low concentrations of arginine found not to provide sufficient substrate for nitric oxide production in hypotensive animals. A low concentration of arginine for purposes of the present invention is defined as less than or equal to about 0.1% arginine in the feeding formulation ready to be administered to the patient. Most preferably, the formulation may include between about 0.01% to about 0.1% arginine.

An additional most preferred embodiment of the claimed invention is essentially arginine free. In one particularly defined embodiment of the essentially arginine-free formulation, the amino acids ornithine and citrulline are included. Ornithine and citrulline contribute to the urea cycle substrate requirements of the animal. Where ornithine and citrulline are included in the formulation, the concentration of these ingredients most preferred comprise about 0.1-0.2% (or 1-2 g/l) ornithine and about 0.1% (or 1 g/l) citrulline.

Table 5
Arginine-Free Formulation Mixture of Amino Acids

Amino Acid	2X Concentrate (mg/100 ml)	Final Feeding Concentration (g/l)
Isoleucine	600	3
Leucine	1,000	5
Lysine	1,000	5
Methionine	200	1
Phenylalanine	300	1.5
Threonine	400	2
Tryptophan	200	1
Valine	500	2.5
Alanine	900	4.5
Histidine	300	1.5
Proline	700	3.5
Serine	400	2.0
Tyrosine	450	2.0
Glycine	800	4.0
Aspartic acid	600	3
Ornithine	400	2

The ornithine content described for the formulation above may be omitted and replaced with citrulline at a concentration of about 2 g/l. The amino acids concentrate (2X0 is mixed with a pharmaceutically acceptable diluent, such as for example, a glucose solution in a proportion of 1 to 1 (1 part amino acid solution to 1 part of a dextrose solution). In addition, trace elements, vitamin supplements and essential salts (Na^+, K^+, PO_4^-, Ca^{++}, Mg^{++}) may be added.

The anti-hypotensive formulations as part of a method for treating or preventing hypotension may be administered as a parenteral nutritional formulation

according to parenteral feeding methods well known to those of skill in the medical arts.

In practicing the claimed method, a physiological benchmark will be referred to in reference to determining at what point the administration of the arginine-free formulation should be terminated. For example, the patient's systolic blood pressure level may be monitored so as to determine when the patient has reached a physiologically acceptable level. A return to normal systolic pressure may then be used to indicate the point at which nitric oxide production was being reduced, and had escaped risk of a greater reduction in peripheral vascular resistance or arterial blood pressure.

As generally defined according to the claimed method, hypotension (low blood pressure) is defined as an adult human systolic blood pressure level of less than about 100 mm Hg. A physiologically acceptable systolic blood pressure in an adult human is at least about 100 mm Hg systolic blood pressure.[40]

It has been observed that serum arginine levels increase upon the administration of a standard TPN formulation.[41] Therefore, by eliminating arginine as an ingredient in a TPN formulation, the inventors propose that serum arginine levels will be significantly reduced in patients receiving such an arginine-free TPN formulation as compared to similarly situated patients whom had instead been receiving a standard TPN solution. A standard TPN solution which includes greater than about 0.1% arginine would not be expected to constitute a hypotensive formulation.

EXAMPLE 7
IN VIVO EFFECTS OF NITRIC OXIDE INHIBITOR NAA ON IL-1 INDUCED HYPOTENSION

The present example is provided to demonstrate the utility of NAA (N^{ω}-amino-L-arginine) and other nitrogen analogs for increasing blood pressure, most particularly in a cytokine-induced hypotensive animal.

Effect of NAA on IL-1-Induced Hypotension

As summarized in Table 6 and illustrated in Fig. 1, anesthetized dogs (n = 4) given IL-1 (50 μg/kg) developed a progressive, moderately severe hypotension, with MAP decreasing from a baseline of 111 ± 7 mm Hg to 80 ± 2 mm Hg within about 3 hours. The IL-1-induced hypotension was rapidly reversed by the intravenous administration of a single bolus dose of NAA (20 mg/kg, 88 μmol/kg); MAP increased by 46 ± 9 mm Hg (58%) to 125 ± 8 mm Hg. In all cases, MAP following NAA administration was significantly higher than the pre-IL-1 baseline MAP (average increase = 14.3 ± 7.7 mm. In control dogs not given IL-1, administration of NAA also increased MAP above baseline; the average increase in MAP was 20.1 ± 3.8 mm Hg, a value not significantly different from the increase above baseline observed in the dogs given IL-1 (p = 0.254). Thus, whereas NAA had a much greater pressor effect in dogs previously made hypotensive by administration of IL-1, the final MAP achieved after NAA administration was similar in control and IL-1-treated dogs.

Specificity of the NAA pressor effect in both IL-1-treated and control dogs was demonstrated by its rapid and complete reversal by L-arginine (400 mg/kg, 2.3 mmol/kg); after arginine administration, MAP averaged 66 ± 27 mm Hg in IL-1-treated dogs, but it was essentially at baseline levels in control dogs. In other studies with IL-1 and other cytokines (e.g., TNF and IL-2), the

present inventors determined the duration of the NAA pressor effect in the absence of L-arginine. Single injections of NAA (20 mg/kg) in cytokine-treated dogs were found to maintain MAP at baseline or higher levels for up to about 60 minutes; second and third injections of NAA (20 mg/kg) had longer effects and maintained physiologically acceptable MAP readings for up to 7 hours (Figure 8).

The central role of vasodilation in IL-1-mediated hypotension is exemplified by the observation that the decrease in MAP was associated with a 33.5% decrease in systemic vascular resistance (Table 6). Following administration of NAA, systemic vascular resistance increased in parallel with the restoration of MAP; systemic vascular resistance values after injection of NAA averaged 43% higher than baseline systemic vascular resistance measurements, although there was significant animal-to-animal variation. Following administration of arginine, systemic vascular resistance decreased substantially in all IL-1-treated animals (average decrease = 61%). In control dogs, the NAA-mediated pressor effect was also associated with an increase in systemic vascular resistance.

Cardiac output was noted to decrease by 29.2% (P = .046) in dogs treated with NAA alone. This decrease was completely reversed by the subsequent administration of L-arginine (Table 6). In dogs treated with IL-1, the cardiac output increased slightly, but not significantly (P = .371). Administration of NAA caused a 25.2% reduction in cardiac output compared with that obtained after the administration of IL-1 (P = .037); however, this value was not statistically different from the baseline cardiac output (1.93 versus 2.25 L/minute; P = .204). These changes were reversed by administration of

L-arginine (Table 6). None of the compounds administered caused a significant change in heart rate.

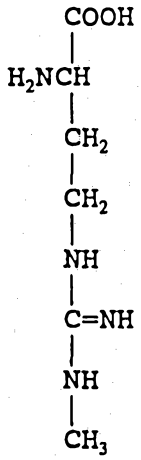
TABLE 6 - Nitric Oxide Synthase Inhibition in Rats

Dog	Mean arterial pressure, mm Hg	Heart rate, beats per minute	Cardiac output, L/min	Systemic vascular resistance, dynes-scc/cm^5
Control (n = 3)	Baseline, 107.9 ± 28.1	113.5 ± 15	2.43 ± 0.88	3649 ± 651
	After NAA, 128.1 ± 29.5	94.3 ± 1	1.72 ± 0.74	6271 ± 1082
	After arginine, 119.1 ± 6.7	107.5 ± 22	2.35 ± 0.65	4116 ± 447
IL-1-treated (n = 4)	Baseline, 111.1 ± 6.7	103.6 ± 16	2.25 ± 0.25	3747 ± 492
	After IL-1, 79.7 ± 1.5	102.1 ± 14	2.58 ± 0.70	2491 ± 756
	After NAA, 125.4 ± 8.4	97.1 ± 23	1.93 ± 0.48	5366 ± 1918
	After arginine, 66.0 ± 26.9	95.0 ± 15	2.20 ± 0.68	2087 ± 937

* Dogs were anesthetized and treated as described. Initial physiological parameters were monitored until constant (baseline). Control dogs were then given NAA (20 mg/kg) in a single bolus injection over 30 seconds. After MAP had stabilized for a least 20 minutes, L-arginine (400 mg/kg) was given. The IL-1-treated dogs were given IL-1 (50 μg/kg) intravenously for 3 minutes and were continuously monitored; the values shown as "After IL-1" represents readings taken at the nadir of MAP, just prior to administration of NAA. Blood pressure recovered within a few minutes of NAA administration; the values shown as "After NAA" represent readings made within 10 minutes of NAA injection. L-Arginine (400 mg/kg) was given for 5 minutes, and physiological determinations were made 10 minutes later. Data reported are mean ± s.d.

EXAMPLE 8
NMMA *IN VIVO* AND BLOCKAGE OF TNF-INDUCED HYPOTENSION

The present example is provided to demonstrate the *in vivo* utility of nitric oxide inhibitors, such as NMMA, for inhibiting hypotension. More specifically, the present example demonstrates that hypotension associated with the administration of TNF in the dog can be blocked by subsequent administration of NMMA. Furthermore, this increase in blood pressure in hypotensive animals is demonstrated to be reversible upon the administration of arginine. NMMA in its free base form has the structural formula:



Furthermore, this inhibition of hypotension can be reversed by administration of an excess of arginine. These results show that NO· is the mediator of hypotension induced by TNF. Furthermore, activation of NO· synthesis may be involved in the pathogenesis of septic shock. This study also demonstrates the hypertensive action of arginine in a TNF-treated animal previously made normotensive by administration of NMMA.

Reagents

Recombinant human TNF, specific activity 2 x 10^7 units/mg, was from the Dainippon Chemical Corporation, Tokyo, Japan. TNF was administered at a dose of 10 µg//kg in a volume of 10 ml of phosphate buffered saline containing 17 mg/ml of dog albumin. NMMA was synthesized by adaptation of the method of Corbin and Reporter (Anal. Biochem. 57: 310-312, 1974), which reference is specifically incorporated herein by reference for this purpose. The NMMA was dissolved in 5 ml of phosphate-buffered saline for administration at a dose of 20 mg/kg. Arginine was obtained from Sigma Chemical Company, St. Louis, Mo.

Animals

Four conditioned mongrel dogs, 2 males and 2 females, weighing 28 to 30 kgs, were studied. Care of the animals were in accordance with the recommendations of the American Association for Accreditation of Laboratory Animals [DHEW(DHHS) publication no. (NIH) 78-23, revised, 1978]. On the day of the experiment, the dogs were fasted overnight. They were anesthetized with phenobarbital (25 mg/kg). The animals were then intubated orally with a #10 fr. endotracheal tube and ventilated with a Harvard pump ventilator at a rate of 14 breaths per minute and a tidal volume of 15 ml/kg. An arterial line was percutaneously placed in the femoral artery on the day of the experiment.

Physiological measurements

Mean (electronic) and phasic systemic arterial pressures (SAP) were continuously recorded on a Hewlett-Packard recording system (model 7758B) using strain gauge manometers (Hewlett-Packard model 1290A) which were

connected to the arterial line. Heart rate (HR) was determined from an EKG tracing and continuously recorded on the Hewlett-Packard recording system. Oxyhemoglobin saturation (SaO_2) was obtained using a pulse oximeter (BIOX 111, Boulder, CO). Continuous time-series records of SAP, HR, and SaO_2 were obtained using a Lab Master analog-to-digital convertor (16 channel, 12 bit, 30 kHz; Scientific Solutions, Inc.) sampling at 55 Hz and storing the 6 sec averages on a magnetic disk.

NMMA was found to reverse the hypotension associated with the administration of TNF (Figure 3). The pressor effect of NMMA occurred rapidly (within 2 minutes) and could be antagonized by administration of an excess of L-arginine. The antagonism of the NMMA pressor effect was stereospecific for the L-form of arginine.

The data shown in Figure 3 is representative of several animal experiments. There were some variations noted in the degree of hypotension as well as the time of onset of hypotension after TNF administration. An example of severe hypotension resulting from administration of TNF is shown in Figure 4a. Ten μg TNF/kg body weight was intravenously administered at the ten minute time point; 4.4 mg NMMA/kg at about 52 minutes; and 3 g L-arginine at about 63 minutes. The onset of hypotension was found to occur between 30 to 60 minutes after TNF. The SAP dropped rapidly from 106 to 36. The administration of NMMA resulted in the rapid increase in blood pressure to an SAP of 116. This represents an 80 mm Hg increase in blood pressure after administration of NMMA.

The administration of NMMA alone to untreated dogs (n=3) was also tested. Within 2 minutes after NMMA infusion, the blood pressure increased by 12 mm Hg. This was followed by a compensatory decrease in the HR with a

return of the BP to baseline. The NMMA-induced bradycardia lasted 31 minutes. This response was not observed in animals which had been previously treated with TNF (Figure 4b). Subsequent administration of L-arginine reversed these small changes observed in systemic arterial pressure. In a second control study nitroglycerin was infused at a rate of 28 μg/kg/minute, IV, to lower the blood pressure to the same level as that observed with tumor necrosis factor (Figure 4c). After administration of NMMA in nitroglycerin infused dogs, the blood pressure increased only 14 mm. Subsequent administration of L-arginine reversed this modest effect.

The administration of L-arginine to cytokine treated dogs reversed the antihypotensive effects of NMMA. Blood pressure was not affected by the administration of L-arginine to previously untreated normotensive dogs.

The dose-limiting toxicity of TNF administered to patients is hypotension. These experiments support that proposition that NO·, also known as EDRF, is the mediator of the hypotension. Furthermore, these hemodynamic changes can be antagonized by an N^{ω}-substituted arginine derivative and subsequently restored by the addition of excess arginine, supporting a role for arginine as the substrate for NO· synthesis. The present inventors have shown that NMMA can increase the resting blood pressure in the guinea pig. Therefore, NO· may play a role in normal arterial pressure homeostasis. This also appears to be true in the dog.

The pressor response to NMMA is much more dramatic in dogs with TNF-induced hypotension than in normotensive dogs. This suggests that TNF induced hypotension is due to an excess production of a vasoactive factor (i.e., NO·) which acts to regulate normal resting blood pressure.

TNF is also involved in the development of the toxicity observed in septic shock. Septic shock is most commonly caused by endotoxin, a component of the cell wall of Gram negative organisms. The administration of anti-TNF antibodies after TNF exposure does not protect against hypotension. However, administration of anti-TNF antibodies may protect against hypotension where administered before TNF exposure. This implies that endotoxin may induce other mediators of hypotension. The results presented herein indicate that NO· is the true mediator of that response.

EXAMPLE 9
L-NMA *IN VIVO* AND ENDOTOXIN-INDUCED HYPOTENSION

This example is provided to demonstrate the utility of nitric oxide synthase inhibitors, such as the arginine analog L-NMA, in treating hypotension in an animal.

In the present study, the effect of L-NMA on endotoxin-induced shock in dogs was examined. The present findings indicate that NO· is an important mediator of endotoxin-induced hypotension and that inhibitors of NO· synthesis should be of value in the treatment of septic shock.

Reagents: Nω-Methyl-L-arginine was synthesized as previously described by Corbin, *et al.* (1974) (Nω-Methylated Arginines: Convenient Preparation of Nω-Methylarginines, *Anal. Biochem.*, 57, 310-312), which reference is specifically incorporated herein by reference for this purpose, and purified by crystallization as the monoflavianate salt. A solution of the free amino acid was obtained by stirring a suspension of the salt with Dowex-1 (OH); after neutralization with HCl, the concentration of L-NMA was determined by amino acid analysis using the crystalline monoflavianate salt as standard. Endotoxin (Escherichia

coli; B0128:B12) and all other reagents were purchased from Sigma Chemical Company, St. Louis, Missouri. Nitroglycerin was purchased from DuPont Pharmaceuticals, Wilmington, D.E.

Animals: Studies were carried out on 12 conditioned mongrel dogs (9 males and 3 females) weighing 22-32 kg (avg=25.3 kg). Animal care was in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care, and met all standards prescribed by the Guide for the Care and Use of Laboratory Animals (Guide for the Care and Use of Laboratory Animals (1978) Dept. of Health, Education and Welfare, Washington, D.C. (Publ. No. 78-23). Animal protocols were approved by The University of Texas Animal Welfare Committee. The dogs were fasted overnight prior to the day of experimentation. They were anesthetized with sodium pentobarbital (25 mg/kg I.v.). Dogs were then endotracheally intubated and ventilated with a piston-driven respirator (Harvard instruments) using room air at a tidal volume of 15 ml/kg and at a rate of 12 to 14 breaths per minute, adjusted to achieve a normal arterial pH and pCO_2 (Instrumentation Laboratories 1L1302 pH/Blood Gas Analyzer). Catheters were placed percutaneously into the femoral and pulmonary arteries; In the latter, a flow-directed thermal-dilation catheter was used (Abbott Critical Care Systems).

Physiologic measurements: Mean SAP and heart rate were continuously monitored (Parametron 7048 Monitoring System, Roche) and stored on a magnetic disk using an analog-to-digital converter (Scientific Solutions, Inc.). Cardiac output (CO) was determined as the mean of six measurements by thermal-dilution. Systemic vascular resistance was calculated as (SAP X80)/CO and expressed as dynes-sec/cm^5.

Protocol: After the blood pressure and heart rate stabilized, endotoxin (40 μg/kg, in 10 ml of phosphate-buffered saline (PBS), pH 7.4) was infused i.v. over 2 minutes. This dose of endotoxin typically induces severe and often lethal cardiovascular collapse in the dog. Blood pressure was monitored, and when either SAP fell below 60 mm Hg or a stable nadir in systemic arterial pressure (SAP) was maintained for 10 minutes, L-NMA was administered (20 mg/kg in 5 ml of PBS i.v. over 1 min.). In most experiments, L-arginine (400 mg/kg in 20 mi PBS) was administered ten minutes later by i.v. infusion over 2 minutes. In control experiments, dogs without prior exposure to endotoxin received L-NMA alone. To simulate the hypotension observed in dogs receiving endotoxin, one group of dogs received a continuous i.v. infusion of nitroglycerin (2 mg/ml) at a rate adjusted to maintain the SAP at 60-70 mm Hg. Nitroglycerin-treated dogs then received L-NMA (20 mg/kg) and 20 minutes later L-arginine was administered (400 mg/ml).

Statistics: Statistical significance was evaluated using Student's test and either a one-tailed or two-tailed analysis as appropriate for comparisons.

A representative blood pressure tracing which depicts the effect of endotoxin on systemic arterial pressure in the anesthetized dog is shown in Figure 5. Cardiovascular parameters for this and 3 additional dogs are summarized in Table 7.

Table 7
Hemodynamic Effects of L-NMA during Hypotension

Type of Evaluation	Systemic Arterial Pressure (mmHg)	Heart Rate (beast/min)	Cardiac Output (L/min)	Systemic Vascular Resistance (dynes-sec/cm^5)
Study 1: Endotoxin-treated (n-4)				
Baseline	128.3 ± 9.4	119.5 ± 6.0	2.99 ± 0.32	3565 ± 454
After Endotoxin	59.5 ± 3.1**	124.0 ± 7.6	2.17 ± 0.44	2403 ± 352
After L-NMA	107.3 ± 9.6**	123.3 ± 4.8	2.03 ± 0.32	4462 ± 552**
After L-Arginine	128.3 ± 9.4	119.5 ± 6.0	2.99 ± 0.32	3565 ± 454
Study 2: Nitroglycerin-treated (n=3)				
Baseline	129.3 ± 10.2	143.7 ± 12.1	3.14 ± 0.21	3294 ± 74
During Nitroglycerin	64.7 ± 2.7**	137.3 ± 5.0	2.72 ± 0.27	1924 ± 132**
After L-NMA	81.8 ± 3.5*	191.7 ± 35.0	3.85 ± 0.80	1858 ± 399
After L-Arginine	57.0 ± 13.0	148.7 ± 19.9	5.15 ± 1.08	1088 ± 491

For study 1, dogs were anesthetized, instrumented, and baseline cardiovascular measurements were recorded (Pretreatment). Endotoxin (40 ug/kg) was then administered and cardiovascular parameters were monitored. When blood pressure either reached a stable nadir or declined below 60 mm Hg (After endotoxin), L-NMA (20 mg/kg) was administered, and cardiovascular parameters were again determined (After L-NMA). After an additional ten min, L-arginine (400 mg/kg) was administered and cardiovascular measurements were determined 2 min. later (After L-Arginine). Results are reported as means ± S.E., (n=4). Study 2 was carried out similarly, except that endotoxin was not administered. Instead, dogs received a continuous infusion of nitroglycerin (2 mg/ml) titrated to maintain SAP AT 65 mm Hg, (n=3). Asterisks indicate statistically significant difference (*$p < 0.005$, **$p < 0.001$) from the immediately proceeding condition.

ET (40 μg/kg) produced a marked decrease in blood pressure within 120 min. (ΔSAP=-69 ± 16 mm Hg, $p<0.05$). Untreated, this dose of endotoxin typically causes lethal

cardiovascular collapse in the dog. L-NMA largely reversed the hypotension within 1.5 minutes, increasing SAP by 47.8 ± 6.8 mm Hg ($p<0.01$) and SVR by 2060 ± 338 dynes-sec/cm^5 ($p<0.01$); HR and CO were unchanged (Table 7). L-arginine reversed the effect of L-NMA and restored the endotoxin-induced hypotension, decreasing both SAP ($p<0.0.01$) and SVR ($p<0.01$) to values similar to those observed before administration of L-NMA. As illustrated in Figure 5, after L-arginine, blood pressure decreased to similar levels compared to those observed prior to L-NMA administration. Figure 5 shows the time course of changes in mean systemic arterial pressure (SAP) in a pentobarbital-anesthetized dog following the i.v. administration of endotoxin (ET), Nω-methyl-L-arginine (L-NMA), and L-arginine (L-Arg). Data from this and additional experiments are summarized in Table 7.

In view of the potential clinical use of NO·-synthesis inhibitors in endotoxin- and cytokine-induced shock, it is important to establish that L-NMA can provide long-term reversal of hypotension. It was found that a single i.v. dose of L-NMA (20 mg/kg) restored normal blood pressure for 30-60 minutes. If an additional dose of L-NMA (20 mg/kg) was given when the blood pressure began to decrease again, normal blood pressure could be maintained for at least 2 hours in the endotoxin-treated dog. Results of a typical study are shown in Figure 6. The maintenance of normal blood pressure continued to be dependent on L-NMA even after 2 hours since L-arginine could still restore endotoxic hypotension at this time (i.e., a decline in blood pressure to less than 45 mm Hg). Figure 6 shows the time course of changes in mean systemic arterial pressure (SAP) in a pentobarbital-anesthetized dog following the i.v. administration of endotoxin. After 53 min. blood pressure declined to 47 mm Hg (ΔSAP=-61 mm Hg). Administration of L-NMA (20 mg/kg) resulted in a rapid

reversal of the severe hypotension (73 mm Hg increase in SAP within 10 min). Blood pressure was maintained for 48 min by the first dose of L-NMA then started to decline. A second dose of L-NMA restored the blood pressure to a level equivalent to the first dose and maintained the SAP greater than 100 mm Hg for 2 hrs. To demonstrate than the potential for hypotension was still retained, the effect of L-NMA was reversed with an excess of L-arginine (400 mg/ml). This resulted in a decline in blood pressure to 43 mm Hg (ΔSAP=-77mm Hg).

As shown in Table 8, L-NMA alone had a significant but modest hypertensive effect in control dogs not treated with endotoxin; L-NMA increased SAP by only 24.8 ± 2.7 mm Hg (p,0.01) with an associated increase in SVR (p=0.01), and decreases in heart rate (HR) and cardiac output (CO) that did not reach statistical significance. L-arginine (400 mg/kg) fully reversed the pressor effect of L-NMA.

TABLE 8
HEMODYNAMIC EFFECTS OF L-NMA IN CONTROL DOGS

	Systemic Arterial Pressure (mm Hg)	Heart Rate (beats/min)	Cardiac Output (L/min)	Systemic Vascular Resistance (dynes-sec/cm^5)
Baseline	129.0±10.9	121±17.9	3.54±0.68	3115±347
After L-NMA	153.8±11.4**	82.5±6.1	2.12±0.26	5967±523*

Experiments were as described in Figure 5, except that endotoxin was not administered. Results are reported as means±S.E., (n=4). Asterisks indicate significant differences from baseline (*p,0.005, **p,0.001). L-NMA = Nω-methyl-L-arginine.

In an additional series of experiments, blood pressure was reduced to 65 mm Hg by continuous i.v. infusion of nitroglycerin, a hypotensive agent that forms NO· by an L-arginine and NO· synthase-independent mechanism. Administration of L-NMA (20mg/kg) to those

dogs resulted in only a 17.1±5.0 mm Hg change without significant alteration in HR, CO, or SVR.

The pathogenesis of the cardiovascular collapse that occurs during septic shock is poorly understood. Current treatment includes i.v. fluid administration and use of pressor drugs to increase peripheral vascular resistance and cardiac output. Very recently, endotoxin-binding agents including polymyxin B (Hanasawa, et al., 1989, New Approach to Endotoxic and Septic Shock by Means of Polymyxin B Immobilized Fiber *Surg. Gynecol. Obstet.* 168:232.) and antibodies which neutralize TNF (Tracey, et al., 1987, Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia *Nature* 330:662-664.) have been used in an attempt to modify the sequelae of septic shock. Although the latter approaches may have prophylactic value, there is not evidence that septic shock can be easily or rapidly reversed by removal of endotoxin or TNF.

Therapy of patients already in septic shock requires intervention at secondary and tertiary steps in the cascade of events initiated by endotoxin. Because the development of hypotension and other changes associated with septic shock may depend on complex interactions between cytokines, eicosanoids, PAF, activated complement components, and other factors, it is not surprising that several interventions have been found to be at least partially effective in some animal models.

Inhibitors of prostaglandin synthesis and PAF receptor antagonists are two major classes of compounds that may have therapeutic potential (8-9). Although these agents appear to be effective, they have been tested primarily in animals administered very large doses of endotoxin (e.g., 1 to 40 mg/kg, or about 1000 times larger than the dose used here). The onset of

hypotension occurs within a few minutes in such animals and may not accurately reflect the cytokine-mediated processes characteristic of clinical septic shock.

In the present study with endotoxin and in a previous study with TNF, microgram doses of ET or TNF were administered, and the hypotensive response occurred after a delay of 30 to 90 min.

The present inventors demonstration that dogs given TNF exhibit a severe hypotension that can be substantially reversed by administration of L-NMA suggested that overproduction of NO· is a major factor in TNF-induced shock. The data in Table 8 show that L-NMA has a rapid and strong anti-hypotensive effect in the endotoxemic dog.

The effects of L-NMA on cardiac output and SVR in the four control dogs showed considerable variation. In two dogs, cardiac output decreased markedly ($\Delta \geq 1.5$ L/min.) and calculated SVR increased dramatically ($\Delta \geq 3500$ dynes-sec./cm). In contrast, major changes in cardiac output after L-NMA administration were not seen in any of the ET-treated dogs or in the other two control dogs; in the latter, SVR increased by only about 1400 dynes-sec./cm^5. Although these results suggest the possibility that L-NMA may have a direct effect on cardiac output under control conditions, additional studies are required. It is likely that activation of the arterial baroreceptor reflex mechanism accounts for the L-NMA-induced decrease in HR and CO under control conditions. In support of this view, it was observed that control dogs given phenylephrine at a dose that elevated SAP to a level similar to that produced by L-NMA alone also showed similar decreases in HR and CO. The lack of effect of L-NMA on HR or CO in hypotensive dogs may be because the

level of hypotension was below the range of baroreceptor reflex sensitivity.

In view of the multiple mediators reported to contribute to septic shock, it was the expectation that even complete inhibition of NO· formation could not fully reverse the hypotension of ET-induced shock. Indeed, that blood pressure was not *fully* restored to pretreatment values by 20 mg/kg L-NMA suggests that mediators other than NO· contribute modestly to hypotension in the endotoxemic dog. The possibility that NO· synthesis was not fully inhibited by the administered dose of L-NMA provides an alternative explanation for the failure to fully restore blood pressure to pretreatment levels. Although direct determination of the extent of NO· synthesis inhibition is not possible in vivo, limited dose response studies indicate that L-NMA doses greater than 20 mg/kg do not have a significantly greater pressor effect. The ET-induced hypotension escaping blockade by 20 mg/kg L-NMA may be due to mediators other than NO·. While it may be that long-term inhibition by L-NMA may be self-limited by conversion to L-Arginine (Salvemini, et al., 1990, Immediate Release of a Nitric Oxide-Like Factor from Bovine Aortic Endothelial Cells by Escherichia coli Lipopolysaccharide. *Proc. Natl. Acad. Sci.* 87:2593.), such metabolism would not be expected to diminish the short-term pressor effect of L-NMA which is shown in Figure 5. Nevertheless, the finding that L-NMA restores blood pressure to normal or near normal values indicates that overproduction of NO· is a major, and perhaps *the* major, cause of hypotension in endotoxic shock.

In one study, a single injection of L-NMA (20 mg/kg) was able to reverse endotoxin-elicited hypotension for 30 to 60 min. As shown in Figure 6, normotension could be maintained for at least 2 hours by a subsequent dose of

L-NMA. The long-term reversal of endotoxin-induced hypotension with L-NMA demonstrates the potential clinical utility of this agent. In conclusion, these results suggest that NO· synthesis inhibitors should be of considerable value in the treatment of septic shock. Since administration of arginine reverses the pressor effect of L-NMA (Figure 5, Figure 6), it is clear that high concentrations of plasma arginine antagonise and may completely abrogate the beneficial effects of L-NMA. L-NMA and related nitric oxide synthase inhibitors should therefore be particularly useful in hypotensive animals maintained at ›w plasma arginine levels such as with a low arginine ,etary source (an arginine-free TPN).

EXAMPLE 10
NMMA AND NAA *IN VIVO* AND TNF CYTOTOXICITY

NMMA does not inhibit the anti-tumor activity of TNF and IL-2, *in vitro*. TNF bioactivity was measured by the cytotoxicity towards murine L929 cells, *in vitro*. Addition of NMMA or Nωaminoarginine did not alter the cytolytic effect of TNF towards tumor cells *in vitro* (Table 10).

TABLE 10

Effects of NMMA on the Cytolytic Activity of rh-TNF Against Actinomycin D-Treated L929 Cells

[NMMA] (mM)	TNF Activity (Units/ml)
0	594.5
0.125	536.9
0.250	538.2
0.500	562.4
0.750	404.7
1.0	415.7

Similarly, NMMA did not alter either the proliferation phase or the lytic phase of human LAK cells exposed to IL-2, *in vitro* (Table 10).

TABLE 11
Effects of NMMA on IL-2 Mediated Lymphokine Activated Killer Cell Activity *in vitro*

[NMMA] (mM)	% Target Cell Lysis*
0	66.1 ± 9.5
0.25	63.3 ± 11.8
0.5	67.7 ± 10.8
1.0	59.3 ± 7.5
2.0	75.1 ± 4.1

* % Lysis calculated from the % of release of radioactivity from ^{51}Cr-labeled Raji Target cells minus spontaneous release. Effector cells were human blood lymphocytes cultured for 4 days in the presence of 40 U/ml of IL-2 (E:T=80:1).

Aminoarginine is the most potent inhibitor of nitric oxide production reported thus far. Since NMMA is metabolized to citrulline which can subsequently serve as a precursor for arginine biosynthesis, other arginine

analogs were tested for their ability to inhibit nitric oxide production (Table 12).

TABLE 12
Comparison of the $ED_{50\%}$* values of Nω-Substituted Arginine Analogs

Analog	$ED_{50\%}$
NMMA	336.7
Aminoarginine	109.5
Nitro-L-Arginine	2115
Nitro-D-Arginine	>4500
Nitro-L-Arginine benzyl ester	>1200
Nitro-L-Arginine methyl ester	1826
Nitro-D-Arginine methyl ester	>4500

*$ED_{50\%}$ + The effective dose of drug that inhibited 50% of the nitrite production by murine endothelial cells exposed to Gamma-Interferon (100U/ml) and TNF (500 U/ml) *in vitro*.

The most potent derivative tested was N$^{\omega}$-aminoarginine. Subsequent testing *in vivo*, showed that aminoarginine was as effective as NMMA in reversing the hypotension associated with TNF administration in the dog (Figure 7).

The reversal of ET shock (lethal dose) by Nω-aminoarginine (NAA) for 4 hrs. 38 min. was demonstrated using multiple doses of aminoarginine (NAA). Figure 8 depicts systemic arterial pressure (SAP) versus time (min). ET (2 mg/kg), a lethal dose, was infused over 60 min. and NAA administered at 97, 165, and 374 min. to maintain blood pressure. The animal was survived for 24 hours and then autopsied. No pathological changes were observed in liver, lungs, heart, brain, bowel or kidney.

EXAMPLE 11
ANTI-HYPOTENSIVE ARGININE-FREE TPN FORMULATION

The present example defines an anti-hypotensive TPN formulation of the present invention. This arginine-free formulation is intended to be used to reduce plasma arginine levels in an animal. The reduced arginine levels in the animal will then augment the anti-hypotensive effect of the nitric oxide synthase inhibitors described herein. This combination therapy can thus be used in the treatment of conditions where hypotension is the sole or an attendant symptom. For example, this regimen may be used in the treatment of an animal in septic shock, an animal treated with chemotherapeutic agents which may reduce blood pressure, or an animal which is generally experiencing hypotension due to trauma.

A sterile, non-pyrogenic, stable solution for parenteral administration to a patient having hypotension or at risk of hypotension or systemic shock, particularly those receiving immunomodulatory agents, is prepared from pure crystalline amino acids, which are dissolved in a glucose solution (5% to 20%) in the following concentrations to provide a 2X concentrate TPN or a ready-to-feed TPN formulation, as indicated:

TABLE 13

Amino Acids	2x concentrate mg/100 ml formulation	Final Concentration (Feeding Formulation) g/l
isoleucine	600-800	3-4
leucine	800-1200	4-6
valine	600-800	3-4
phenylalanine	200-400	1-2
methionine	200-400	1-2
lysine	600-800	3-4
histidine	200-400	1-2
threonine	400-600	2-3
tryptophan	100-300	0.5-1.5
tyrosine	50-150	0.25-0.75
alanine	800-1000	4-5
aspartic acid	400-600	2-3
glycine	800-1000	4-5
proline	600-800	3-4
serine	200-400	1-2

To obtain the preferred TPN formulation concentration suitable as a feeding formulation, a volume of 500 ml of the 2X concentrate (defined in Table 13) is mixed with 500 ml of a 50% dextrose solution, for the production of 1 liter of the feeding formulation (i.e., 500 cc of a 2X concentrate of AA and 500 cc of dextrose solution). Most preferably, the dextrose solution is supplemented with a physiologically acceptable concentration of vitamins and minerals.

The TPN of the present methods may also include glutamic acid (400-600 mg/100 ml of a 2x conc., or 2-3 g/l in a final concentration) and/or taurine (50-100 mg/100 mls. of a 2-fold concentrate; 0.25-0.5 g/l final concentration).

The solution is then filter sterilized into appropriate containers for intravenous fluids. To prepare for administration, the volume is then brought to the desired feeding solution concentration with an equal volume of sterile glucose solution. The TPN as a ready to feed formulation is then to be kept cool. The solution may then be administered to a patient intravenously (IV). The pH of the TPN solution must also

be adjusted to a physiologically acceptable pH, between 7.0 and 7.4. The formulation is arginine-free.

PROPHETIC EXAMPLE 12
FORMULATIONS AND METHODS FOR INHIBITING HYPOTENSION AND SEPTIC SHOCK

The present prophetic example provides methods whereby the particularly defined arginine-free formulations described herein may be used in the treatment of patients at risk of developing hypotension or septic shock, or whom may require parenteral nutritional support and are at risk of developing or who have already developed hypotension or septic shock.

The proposed formulations and proposed methods may be used most particularly useful in the clinical management of patients requiring total parenteral nutritional support and receiving immunomodulators. The term "immunomodulator" refers to such agents as interferon, interleukin-2, interleukin-1 and tumor necrosis factor as used in the description of the present invention.

Many of the class of substances recognized as immunomodulators are used as anti-cancer chemotherapeutic agents or immunorestorative agents in cancer patients receiving standard cytotixic drugs. Thus, it is envisioned that the presently described methods would be effective for the clinical management of patients being maintained on parenteral nutritional support and receiving chemotherapeutic agents with immunomodulatory action or immunorestorative action (eg., interleukin-1).

According to the present invention, a method for prophylaxis or treatment of systemic hypotension related to the elevated production of nitric oxide in an animal is provided comprising administering to the animal a non-

hypotensive formulation which is arginine-free comprising a mixture of amino acids in a pharmaceutically acceptable diluent. The formulation is again to be essentially arginine-free.

The formulation is to be administered to the patient until a physiologically acceptable blood pressure in the animal is reached and maintained. For a human, a physiologically acceptable systolic blood pressure level is about 100 mm Hg.

More particularly, the method of the present invention includes an essentially arginine-free formulation comprising a mixture of amino acids. The formulation should be prepared so as to be physiologically suitable as an intravenous hyperalimentation (total parenteral nutrition) solution for patients requiring such solutions.

Stated as a range of concentrations for the most preferred mixture of amino acids, the formulation of the presently disclosed methods and specially tailored arginine-free formulations is defined in Table 14. Most preferably, the proposed concentrations to be included in such a formulation appear in Table 14.

TABLE 14
PREFERRED RANGES OF AMINO ACIDS IN NON-HYPOTENSIVE FORMULATIONS

about 3-4 g/l isoleucine (0.3-0.4%);
about 4-6 g/l leucine(0.4-0.6%);
about 3-4 g/l lysine (0.3-0.4%);
about 1-2 g/l methionine (0.1-0.2%);
about 1-2 g/l phenylalanine (0.2-0.2%);
about 2-3 g/l threonine (0.2-0.3%);
about 0.5-1.5 g/l tryptophan (0.05-0.15%);
about 3-4 g/l valine (0.3-0.4%);

about 4-5 g/l alanine (0.4-0.5%);
about 1-2 g/l histidine (0.1-0.2%);
about 3-4 g/l proline (0.3-0.4%);
about 1-2 g/l serine (0.1-0.2%);
about 0.25-0.75 g/l tyrosine (0.025-0.075%);
about 4-5 g/l glycine (0.4-0.5%); and
about 2-3 g/l aspartic acid (0.2-0.3%).

The formulation may also include ornithine. Where ornithine is part of the particular formulation, it is to be included at a concentration of about 1-2 grams/l of the TPN feeding formulation (about 0.1-0.2% ornithine).

Where the formulation is a parenteral formulation, the mixture should be adjusted so as to be physiologically compatible for parenteral administration.

The described non-hypotensive parenteral nutritional formulations may alternatively include low concentrations of arginine found not to provide sufficient substrate for nitric oxide production in hypotensive animals. A low concentration of arginine for purposes of the present invention is defined as less than or equal to about 0.1% arginine in the feeding formulation ready to be administered to the patient. Most preferably, the formulation may include between about 0.01% to about 0.1% arginine.

An additional most preferred embodiment of the claimed invention is essentially arginine-free. In one particularly preferred embodiment of the essentially arginine-free formulation, the amino acids ornithine and citrulline are included. Ornithine and citrulline contribute to the urea cycle substrate requirements of the animal. Ornithine or citrulline, or both are to be considered optional components of the formulation. These additional ingredients are to be included to support

nutritional requirements of the urea cycle. Where ornithine and citrulline are included in the formulation, the concentration of these ingredients most preferred comprise about 0.1-0.2% (or 1-2 g/l) ornithine and about 0.1% (or 1 g/l) citrulline.

Table 15
Arginine-Free Formulation Mixture of Amino Acids

Amino Acid	2X Concentrate (mg/100 ml)	Final Feeding Concentration (g/l)
Isoleucine	600	3
Leucine	1,000	5
Lysine	1,000	5
Methionine	200	1
Phenylalanine	300	1.5
Threonine	400	2
Tryptophan	200	1
Valine	500	2.5
Alanine	900	4.5
Histidine	300	1.5
Proline	700	3.5
Serine	400	2.0
Tyrosine	450	2.0
Glycine	800	4.0
Aspartic acid	600	3
Ornithine	400	2

The ornithine content described for the formulation above may be omitted or it may be replaced with citrulline at a concentration of about 2 g/l. The amino acids concentrate (2X) is mixed with a pharmaceutically acceptable diluent, such as for example, a glucose solution in a proportion of 1 to 1 (1 part amino acid solution to 1 part of a dextrose solution). In addition,

trace elements, vitamin supplements and essential salts (Na^+, K^+, PO_4^-, Ca^{++}, Mg^{++}) may be added.

The anti-hypotensive formulations as part of a method for treating or preventing hypotension may be administered as a parenteral nutritional formulation according to parenteral feeding methods well known to those of skill in the medical arts.

In practicing the claimed method, a physiological benchmark will be referred to in order to determine at what point the administration of the arginine-free formulation should be terminated. For example, the patient's systolic blood pressure level may be monitored so as to determine when the patient has reached a physiologically acceptable level (defined as about 100 mm Hg). A return to normal systolic pressure may then be used to indicate the point at which nitric oxide production was reduced, and the animal being treated had escaped risk of a greater reduction in peripheral vascular resistance or arterial blood pressure.

As generally defined according to the claimed method, hypotension (low blood pressure) is defined as an adult human systolic blood pressure level of less than about 100 mm Hg. A physiologically acceptable systolic blood pressure in an adult human is at least about 100 mm Hg systolic blood pressure.

It has been observed that serum arginine levels increase upon the administration of a standard TPN formulation. Therefore, by eliminating arginine as an ingredient in a TPN formulation, the inventors propose that serum arginine levels will be significantly reduced in patients receiving such an arginine-free TPN formulation as compared to similarly situated patients whom had instead been receiving a standard TPN solution.

A standard TPN solution which includes greater than about 0.1% arginine would not be expected to constitute a hypotensive formulation within the meaning of the present invention.

PROPHETIC EXAMPLE 13
THERAPEUTIC REGIMEN OF ARGININE-FREE FORMULATIONS AND NITRIC OXIDE INHIBITORS FOR THE TREATMENT OF HYPOTENSION

The present prophetic example is provided to outline a proposed method for treating the hypotension attendant a variety of pathologies, such as septic shock, chemotherapy-related hypotension, etc., in an animal. Most particularly, the herein described methods are provided to outline those most preferred methods for treating hypotension in an animal, such as a human, with the herein described arginine-free parenteral formulations and arginine analogs. The arginine analogs described herein have also been shown by the present inventors to be nitric oxide inhibitors, most specifically nitric oxide synthase inhibitors. Therefore, these terms may be used interchangeably in the description of the present methods and regimens.

One of ordinary skill in the medical arts armed with the *in vivo* and *in vitro* results disclosed herein regarding the effect of lowered physiological arginine in an animal and the effects of various arginine analogs and nitric oxide synthase inhibitors, will be apprised of sufficient teaching to employ the herein described therapeutic regimens and methods to treat hypotension in a patient. Particular physiological guidelines for the adaptation of doses and physiologically compatible solutions for use in a patient may be obtained through reference to a variety of medical text books, including Remington's *Pharmaceutical Sciences* (1990) (Mack Publishing Company, Easton Pennsylvania 18042, (18th edition)). This reference text is specifically

incorporated herein by reference for the purpose of providing physiological and/or pharmacological guidelines to be considered by the clinician in preparing physiologically compatible and non-toxic formulations in treating a hypotensive condition in a patient.

The present inventors have demonstrated that a decrease in the physiological levels of arginine in an animal, particularly in a hypotensive animal will enhance the pressor response to a pressor agent in the animal to provide an increase in blood pressure *in vivo*. In addition, the inventors have demonstrated herein that administration of a variety of arginine analogs, including NMA, NAA and NNA, provide an effective means of increasing blood pressure in hypotensive animals. This effect was demonstrated, even more specifically, in endotoxin-induced hypotensive animals. The studies presented in the present disclosure have also demonstrated that hypotensive animals which were not treated in any manner to reduce physiological arginine levels demonstrated a blunted response to blood pressure increasing agents (pressor agents). These studies, taken together, provide a reasonable scientific expectation that a combination therapeutic regimen which decreased physiological levels of arginine in a hypotensive animal, and which provided for the concurrent administration of a arginine analog capable of increasing blood pressure, or which provided for the administration of such an arginine analog subsequent to an effective decrease in physiological arginine levels, will provide an effective treatment for improving and/or eliminating a hypotensive condition in a patient. A reasonable correlation may be made between the results reported herein using laboratory animals and those responses which may be expected upon treatment of a human patient.

Accordingly, hypotension in a patient may be effectively treated by initially maintaining the patient on a supportive dietary source which is arginine-free. It is contemplated that reduced physiological concentrations of arginine may be obtained in a patient by maintaining the patient on an arginine-free parenteral formulation (TPN). Elimination of arginine from the parenteral formulation will function to decrease available arginine in the animal. As demonstrated in the examples provided herein, a decrease in available arginine levels in a hypotensive animal will function to enhance the response of the animal to a pressor agent. Most preferably, it is contemplated that a sufficiently lowered arginine concentration in the patient may be obtained after the patient has been maintained on the arginine-free parenteral formulation for at least 2-6 hours. The patient should be monitored by the attending physician for a decrease in arginine concentrations. An initial plasma or serum arginine level should be obtained as a baseline from which to gauge relative increases or decreases in physiological arginine levels. Most preferably, plasma or serum levels of arginine in the patient will be reduced to less than 4 μM arginine prior to the administration of an arginine analog to the patient. Lesser reductions in serum arginine will, however, be acceptable and even more preferred in the practice of the described method.

While results between patients will vary, it is contemplated that maintenance of a patient on an arginine-free parenteral formulation for at least 2-24 hours will reduce physiological arginine levels in the patient sufficiently to provide the enhanced presser response with the arginine analog described herein. As used in the present description, the terms arginine analog and nitric oxide inhibitor and nitric oxide synthase inhibitor are used interchangeably.

Upon the reduction of physiological arginine levels in the patient to an acceptable level (defined as between trace and one-half of the original concentration of arginine) an arginine analog, such as NMA, NAA, NNA, or a combination thereof is to be administered to the patient. Most preferably, the arginine analog is to be administered *via* an intravenous route in a single bolus dose. The arginine analog is to be prepared most preferably in a sterile saline solution at a concentration sufficient to provide the patient with a dose of the arginine analog of the between 0.1 mg/kg to about 100 mg/kg. Again, most preferably, the dose of the arginine analog most preferred for use in the herein described method is between about 10 mg/kg to about 30 mg/kg, or about 20 mg/kg.

The patient should be monitored for relative increases or decreases in blood pressure continuously at least once every hour during the treatment, and especially after administration of the arginine analog. The arginine analog of choice most preferred is NMA. Where no increase in blood pressure is obtained upon administration of the arginine analog, the dose of arginine analog should be increased and the increased dose provided to the patient in a subsequent treatment within 30 minutes of the initial arginine analog treatment.

EXAMPLE 14
PREPARATION OF ANTI-ENDOTOXIN ANTIBODIES

The present example is provided to demonstrate a proposed method for preparing anti-endotoxin antibodies to be used in the herein described combination therapeutic regimens and methods with an arginine-free parenteral formulation.

Polyclonal antibodies to endotoxin may be prepared through use of an immunization protocol, wherein an animal, for example a mouse, may be injected with a commercial preparation of endotoxin in an amount sufficient to establish anti-endotoxin antibody titer levels in the animal. By way of example, a commercial source of endotoxin may be obtained from Sigma Chemical Co. (St. Louis, Missouri). Endotoxin includes a polypeptide which is common to both gram negative bacterial and *Esceriachia coli* cell wall materials.

HA-1A (Centoxin) is a human monoclonal IgM antibody that binds to the lipid A domain of endotoxin and is produced by the stable heteromyeloma cell line A6(H4C5) developed by Teng, Kaplan, and Braude. (Teng *et al.* Proc. Natl. Acad. Sci. U.S.A. 82:1790-4, 1985). The Teng *et al.* reference is specifically incorporated herein by reference for this purpose. HA-1A has been shown to bind specifically to many endotoxins and to a broad range of clinical isolates of gram-negative bacteria. In various animal models of gram-negative bacteremia and endotoxemia, the administration of HA-1A after challenge prevents the development of the dermal Shwartzman reaction and death. (Teng *et al.* Proc. Natl. Acad. Sci. U.S.A. 82:1790-4, 1985; Ziegler *et al.* A. Clin. Res. 35:519A, 1987).

HA-1A is produced by continuous-perfusion cell culture and is purified from the supernatant fluid by a series of steps involving selective precipitation and column chromatography. (Ziegler *et al.* N. Engl. J. Med. 324:429-436, 1991). The cell line that produces HA-1A has been tested extensively and has been shown to be free of human viruses. Furthermore, the purification process for HA-1A includes specific viral-inactivation procedures, and tests are performed to confirm the absence of viruses. None of the lots contained

detectable endotoxin in an assay with a sensitivity of 3 pg per milliliter.

Two monoclonal antibodies to endotoxin, E5 and HA-1A, await approval from the Food and Drug Administration. E5, which was developed from murine splenocytes immunized with J5 mutant *E. coli* cells, is an IgM antibody with reactivity to lipid A. (Greenman *et al.*, JAMA 266:1097-1102, 1991). HA-1A is a human IgM antibody (also derived from immunization with J5 mutant cells) that binds specifically to lipid A (Ziegler *et al.*, N. Engl. J. Med. 324:429-436, 1991). Both E5 and HA-1A have been shown to bind to endotoxin from a wide variety of gram-negative bacteria, and both have been evaluated in randomized, double blind, controlled trials.

In an HA-1A study, a single 100-mg intravenous dose of antibody was administered to 262 patients; 281 received placebo. (Ziegler *et al.* N. Engl. J. Med. 324:429-436, 1991). Active treatment produced a 39% decrease in 28-day mortality among the 200 patients with gram-negative bacteremia (37% of all study patients). Treatment benefit extended to the 101 patients with gram-negative bacteremia who were in shock (defined as a systolic blood pressure of <90 mm Hg or the use of vasopressors to maintain blood pressure) at study entry; in this subgroup, active treatment reduced 28-day mortality by 42%. Among patients with gram-negative bacteremia, active treatment increased the organ failure resolution. All evidence of organ failure disappeared within 7 days in 38 of 61 patients (62%) given HA-1A and in 26 of 62 patients (42%) given placebo.

Some investigators have reported negative results with anti-endotoxin core antiserum. Most of their studies were performed in mice. Although the reasons for discrepant results are not fully understood, several

factors may contribute. (McCabe *et al.* J. Infect. Dis. 158:291-300, 1988; Ziegler, E.J. J. Infect. Dis. 158:286-290, 1988). These include the relative resistance of rodents to endotoxin, the need to compromise host defenses severely in order to establish satisfactory animal models of gram-negative infection and endotoxemia, and the rather low affinity of cross-reactive anti-endotoxin core antibodies as compared with type-specific antibodies. Recently, Baumgartner *et al.* (J. Exp. Med. 171:889-96, 1990) reported a lack of protection by a human monoclonal antibody derived from cells isolated from the same original clone as HA-1A, but the antibody was not produced or purified by the laboratory that produced HA-1A. When species resembling humans in endotoxin sensitivity are studied, protection from J5 antibody can be demonstrated (Spier *et al.* Circ. Shock 28:235-48, 1989). The negative results of Calandra *et al.* with J5 immunoglobulin in patients with gram-negative septic shock (Calandra *et al.* J. Infect. Dis. 158:312-9, 1988) may have been due to the absence of IgM in their preparation.

HA-1A has been administered in phase I trials to 15 patients with cancer (Khazaeli *et al.* J. Biol. Response Mod. 9:178-84, 1990) and in unblinded fashion to 34 patients with sepsis, (Fisher *et al.* Crit. Care Med. 18:1311-5, 1990) as well as to the 291 patients who received it in the aforedescribed trial. In all these patients, HA-1A was safe, well tolerated, and nonimmunogenic.

Nitric oxide synthase inhibitors administered concomitantly with the anti-endotoxin antibody may include an arginine analog having inhibitory activity toward nitric oxide synthase as described in previous examples. It is expected that lower doses of the anti-endotoxin antibody will be therapeutically effective when

administered concomitantly or subsequent to an arginine-free parenteral formulation.

EXAMPLE 15
PREPARATION OF ANTI-TUMOR NECROSIS FACTOR ANTIBODIES

The present example is provided to demonstrate a proposed method for preparing anti-tumor necrosis factor antibodies for use in the herein described therapeutic regimens and methods. Either a polyclonal antibody or monoclonal antibody specific for tumor necrosis factor may be prepared according to methods known to those of skill in the art. For preparation of a polyclonal antibody, immunization techniques wherein an animal is immunized with tumor necrosis factor, may be utilized. Alternatively, monoclonal antibodies may be prepared according to standard hybridoma protocols, wherein the spleen of a tumor necrosis factor-immunized animal is fused to an immortal tumor cell line to provide a hybridoma which produces anti-tumor necrosis factor monoclonal antibodies.

The administration of antibodies to TNFα protects against the lethal effects of subsequent endotoxin challenge in animals (Beutler *et al.* Science 229:869-871, 1985; Tracey *et al.* Nature 330:662-664, 1987; Opal *et al.* J. Infect Dis. 161:1148-1152, 1990; Silva *et al.* J. Infect. Dis. 162:421-427, 1990); this protection holds even if anti-TNFα antibodies are administered 30 minutes *after* endotoxin challenge (Hinshaw *et al.* Circ. Shock. 30:279-292, 1990; Bahrami *et al.* *In:* Program and Abstracts of the Second International Congress on the Immune Consequences of Trauma, Shock, and Sepsis: Mechanisms and Therapeutic Approaches; March 6-9, 1991; Munich, Germany, Abstract). Thus, TNFα is believed to play a central role in the development of sepsis, and administration of anti-TNFα antibodies appears to be an attractive method for improving outcome, particularly

when used in conjunction with an arginine-free parenteral formulation.

The efficacy of anti-TNF antibodies for treating hypotension in humans was demonstrated in humans by Exley *et al.* (1990). The anti-TNF antibodies described in Exley *et al.* (1990) therefore constitute a most particularly preferred monoclonal antibody for use in the present invention. This reference is specifically incorporated herein by reference for this purpose. In a phase 1 study, Exley *et al.* (Lancet 335:1275-1277, 1990) administered murine IgG monoclonal antibodies to recombinant human TNFα (CB0006) to 14 patients with severe septic shock. Mean arterial pressure increased markedly after CB0006 antibody infusion, and there were no adverse reactions to treatment. A controlled clinical trial of anti-TNFα antibodies is now under way.

A major potential benefit of monoclonal antibodies to TNFα is that such treatment may improve outcome in both gram-negative and gram-positive sepsis. Several factors may limit the success of this agent, however. First, TNFα levels have been detected in only about one third of patients with septic shock (Marks *et al.* Am. Rev. Respir. Dis. 141:94-97, 1990), possibly because of the short half-life of TNFα in humans (14 to 18 minutes). Thus, anti-TNFα antibodies may be administered too late (or too early) for them to have any effect. Second, several studies have demonstrated that elevated TNFα levels alone are insufficient to produce shock (Silva *et al.* J. Infect. Dis. 162:454-459, 1990; Sun *et al.* Am. J. Pathol. 136:949-956, 1990). Third, anti-TNFα antibodies may not be effective against all causes of sepsis.

However, it is contemplated that anti-TNF antibody, when used in conjunction with an arginine-free parenteral formulation described herein, would provide an effective

method and regimen for the treatment of hypotension, particularly that hypotension attendant septic shock.

PROPHETIC EXAMPLE 16
ANTI-ENDOTOXIN ANTIBODIES AND ARGININE-FREE TPN FOR TREATMENT OF HYPOTENSION IN SEPTIC SHOCK

This example provides interleukin 1 receptor antagonist (particularly IL 1ra) in combination with an arginine-free parenteral formulation nitric oxide synthase inhibitors for treatment of hypotension.

An IL-1 specific inhibitor has been produced from human monocytes (Seckinger *et al.* J. Immunol. 139:1541-1545, 1987; Arend *et al.* J. Immunol. 134:3868-3875, 1985); this inhibitor blocks the binding of IL-1 to its cell surface receptors (Seckinger *et al.* J. Immunol. 139:1541-1545, 1987). The inhibitor has been cloned, and sequence analysis reveals 40% conserved amino acid homology with IL 1ß. It has been renamed the IL-1 receptor antagonist (IL 1ra)(Hannum *et al.* Nature 343:336-340, 1990; Eisenberg *et al.* Nature 343:341-346, 1990; Carter *et al.* Nature 344:633-638, 1990), and it competes with IL-1 for occupancy of surface receptors without agonist effects.

Wakabayashi *et al.* (FASEB Journal 5:338-343, 1991) have blocked endogenous IL-1 activity by pretreatment with human recombinant IL 1ra in a model of *E. coli*-induced shock in rabbits. The IL 1ra-treated group received IL 1ra intravenous injection as a 10-ml bolus (10 mg/kg) at t = -15 min, followed by a constant rate (15 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 4 h. In saline-treated controls, hypotension was sustained for 4 h and death occurred for two of five rabbits; in rabbits treated with the IL 1ra, however, blood pressure was only transiently decreased, returned to pre-*E. coli* levels, and no deaths occurred. The associated leukopenia was also reduced by treatment

with the antagonist ($P < 0.05$). Histological examination of lung tissues showed reduced infiltrating neutrophils in the IL 1ra treatment group. Despite the attenuated responses in animals treated with the IL 1ra, circulating TNF and IL 1 levels were nearly identical in both groups. The specific blockade of IL 1 at the receptor level demonstrates an essential role for this cytokine in the pathogenesis of septic shock.

An aspect of the present invention is the concomitant administration of the IL 1ra in combination with an arginine-free parenteral formulation in the treatment of hypotension. The Wakabayashi *et al.* (1990) article is specifically incorporated herein by reference for the purpose of describing a method for preparing an IL-1 receptor antagonist which may be used in the practice of the present invention.

PROPHETIC EXAMPLE 17
ANTI-ENDOTOXIN ANTIBODIES AND ARGININE-FREE TPN FOR TREATMENT OF HYPOTENSION IN SEPTIC SHOCK

The present example is provided to demonstrate the proposed use of a therapeutic regimen of an arginine-free parenteral formulation and anti-endotoxin antibodies in the treatment of hypotension. More specifically, it is proposed that the present methods and compositions may be used in the treatment of hypotension in patients exposed to endotoxin and/or who have developed septic shock.

The earliest possible intervention in endotoxin (septic) shock involves the use of antibodies directed against endotoxin itself or against cytokines participating in the cascade initiated by endotoxin (i.e., tumor necrosis factor and IL-1) Although antibodies are of no use in studies such as those of Ochoa *et al.*, (1992) *J. Natl. Cancer Inst.*, 84:854-867, where a cytokine is used therapeutically, antibodies show

some clinical activity in septic shock (Ziegler *et al.* (1991) *N. Eng. J. Med.* 324:429-437); Baumgartner *et al.* (1990) *J. Exp. Med.* 171:889-896; Calandra *et al.* (1988) *J. Infect. Dis.* 158:312-319. The advantages of antibody therapy are that all adverse effects of endotoxin can, in principle, be avoided and that constitutive nitric oxide synthase is not inhibited. Antibodies should most preferably be given before nitric oxide synthase is induced. Once induced, nitric oxide synthase may remain active for 24-48 hours. In a clinical setting, NO mediated shock cannot be anticipated with the necessary degree of certainty.

Antibodies directed against endotoxins will be prepared as described herein using immunization protocols of an animal injected with endotoxin, or by standard hybridoma technology well known to those of skill in the art. One potential method for the preparation of these antibodies is described herein. The most preferred anti-endotoxin antibody for use in the invention is HA-1A, previously described. A commercial source of endotoxin is available from suppliers such as Sigma Chemical Co. (St. Louis, Missouri).

The anti-endotoxin antibody would preferably be administered to the patient concurrently with the administration of an arginine-free TPN. The arginine-free TPN is described in Example 1, and most preferably will include citrulline, ornithine, or both, to even further satisfy urea cycle requirements in the animal.

A therapeutically effective concentration of the anti-endotoxin antibody will be determined on the basis of patient response as an improvement of the hypotensive condition. An improvement in the hypotensive condition as defined for purposes of describing the present invention, is demonstrated by an increase in systolic

pressure to at least 100 mm Hg in the patient. A systolic blood pressure of at least 100 mm Hg is defined for purposes of describing the present invention as a normotensive condition. The antibodies are most preferably to be administered before nitric oxide synthase induction to be fully effective.

PROPHETIC EXAMPLE 18
INTERLEUKIN-1 RECEPTOR ANTAGONISTS AND ARGININE-FREE TPN FOR TREATMENT OF HYPOTENSION

The present example is provided to outline a proposed method for the treatment of hypotension in an animal, particularly that hypotension attendant exposure to endotoxin or septic shock, through the use of an arginine-free parenteral formulation and an interleukin-1 receptor antagonist. The most preferred interleukin-1 receptor antagonists for use in conjunction with the present invention may be obtained from, or prepared according to the method outlined in Wakabayashi *et al.* (1991), which reference is specifically incorporated herein by reference for this purpose.

By way of example, the specific interleukin-1 receptor antagonist to be used is IL 1ra. This interleukin-1 receptor antagonist is described in Wakabayashi *et al.* (1991)[50], and is most preferably to be administered *via* a bolus intravenous infusion of between about 1 mg/kg to about 100 mg/kg, with the most preferred dose being about 10 mg/kg.

The arginine-free parenteral formulation is to be prepared as described herein. A patient having a systolic blood pressure of less than about 100 mm Hg will be targeted for the present treatment. Such a patient is to be placed on a continuous feed of an arginine-free formulation which includes a mixture of essential and nonessential amino acids as described herein. The

formulation, in one embodiment, may be supplemented with between 1-2 g/l ornithine and/or between 1-2 g/l citrulline. The patient is to be maintained on the arginine-free TPN concurrently with the interleukin-1 antagonist. Blood samples are to be obtained from the patient and arginine levels in the serum or plasma fraction are to be determined. The patient is to be given an intravenous bolus dose of interleukin-1 receptor antagonist, most preferably IL-1 ra. This particular interleukin-1 receptor antagonist is described in Wakabayashi *et al.* (1991)[50], which reference is specifically incorporated herein by reference for this purpose.

Tachycardia (>90 beats per minute in the absence of beta-blockade) and tachypnea (respiratory rate >20 beats per minute or the requirement of mechanical ventilation) have been characterized as signs of systemic toxicity (Ziegler *et al.* (1991) *The New England Journal of Medicine*, 324(7):429-436). With the combination therapeutic regimen described herein, the treated patient may also be free of these systemic toxicity symptoms as well as exhibiting normotensive systolic blood pressure.

The blood pressure of the patient may be monitored, for example using a cuff-blood pressure monitoring device, after the interleukin-1 receptor antagonist is administered. The patient is to be maintained on the continuous parenteral feed of the arginine-free TPN both before and after the antagonist is administered. A return to normotensive blood pressure levels (at least 100 mm Hg systolic blood pressure) may result in the patient upon treatment according to the claimed method. Reduction in serum/plasma arginine levels in combination with the antagonist may provide relief from other signs of systemic toxicity, unlike previous reports using the antagonist alone (IL 1ra).

PROPHETIC EXAMPLE 19
ANTI-TUMOR NECROSIS FACTOR AND ARGININE-FREE TPN FOR TREATMENT OF HYPOTENSION

The present example is provided to demonstrate one preferred method by which the herein described therapeutic regimen and methods may be employed for the treatment of hypotension.

Anti-tumor necrosis factor antibodies may be prepared as described by Calandra *et al.* (1991), *In*: Bacterial Endotoxins: Cytokines Mediators and New Therapies for Sepsis, pp. 141-159), which reference is specifically incorporated herein by reference for this purpose. By way of example, such anti-tumor necrosis factor antibodies include a polyclonal anti-TNF antibody described by Beutler *et al.*, which reference is specifically incorporated herein by reference for this purpose (Beutler *et al.* (1985), *Science*, 229:869-871). An anti-TNF monoclonal antibody may be used for the herein described methods as well, and is a more particularly preferred aspect of the claimed invention. Such a monoclonal antibody for TNF is described by Tracey *et al.* (1987), *Nature*, 330:662-664. The Tracey *et al.* article is also incorporated herein by reference for the purpose of describing a method for preparing the anti-TNF monoclonal antibodies which may be used in conjunction with the herein claimed combination therapeutic regimens and methods. Such an anti-TNF monoclonal antibody preferred is CB0006.

A patient is first to be identified as having hypotension, a condition which is described for purposes of the present invention as a systolic blood pressure of less than about 100 mm Hg. Once a patient has been determined to have a hypotensive condition, he or she is to be started on an parenteral formulation which is arginine-free. The arginine-free TPN is defined

compositionally herein. This formulation may also include citrulline and/or ornithine so as to insure metabolic requirements of the urea cycle in the animal. The animal or patient is to be maintained on the arginine-free TPN until serum or plasma arginine levels have been reduced. For this reason, the patient or animal is to be monitored continuously for changes in arginine concentrations in serum and/or plasma samples.

A bolus dose of the anti-TNF antibody, most preferably a monoclonal anti-TNF antibody as described in Exley *et al.* (1989) (Murine monoclonal antibody to recombinant human tumor necrosis factor in the treatment of patients with severe septic shock. Abstract No. 324. Program and abstracts of the 29th interscience conference on anti-microbial agents and chemotherapy, 155) is administered to the patient simultaneously with the arginine-free parenteral formulation. Thus, during administration of the anti-TNF antibody, the patient and/or animal is to be maintained on the arginine-free parenteral formulation. Upon such treatment, the patient and/or animal may demonstrate an increase in systemic blood pressure levels to normotensive levels (i.e., at least about 100 mm Hg).

Treatment of patients with severe systemic shock with a murine monoclonal antibody to recombinant human TNF (anti-rhTNF) was reported in the Exley *et al.* (1989) study, with a reversal of hypotension in nine of ten patients. However, 50% of these patients reported died within 7 days of attaining normotensive levels. Such a mortality rate is reported by those authors as relatively the same as mortality rates in patients with septic shock receiving no treatment (see Calandra *et al.* (1988). Therefore, no protection against death is observed when anti-TNF antibodies are used alone after an infectious challenge. Administration of an arginine-free total

parenteral formulation to the patient prior to and concurrently with the antibody treatment may provide for an enhancement in patient survival and improvement in patient mortality.

EXAMPLE 20

The present example demonstrates the effects of interleukin-1 receptor antagonists on the induction of nitric oxide synthase by IL-1 or IL-1 plus IFN-α. While the results demonstrate *in vitro* results, the data is also indicative of those effects to be expected from the use of interleukin-1 receptor antagonist *in vivo*.

This example demonstrates inhibition of IL-1-α-induced nitric oxide synthase in vascular smooth muscle.

Materials

Human recombinant, IL-1-α (hereinafter referred to as IL-1; specific activity, 2 x 10^9 lymphocyte-activating factor units/mg) was produced by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and provided by the National Cancer Institute. Human recombinant Il-1 receptor antagonist was produced by Synergen (Boulder, Co.). Rat interferon-γ (IFN-γ) was obtained from Amgen Biologicals (Thousand Oaks. Ca.) Except where indicated, all biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cell culture media and reagents, unless otherwise noted, were from Whittaker Bioproducts (Walkersville, Md.).

Cell Culture

Mouse A375 melanoma cells were provided by Dr. E. Kleinerman, The University of Texas M.D. Anderson Cancer Center. Cells were maintained in Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1) containing 10 mM HEPES buffer (pH 7.4) and 10% fetal bovine serum. All

tissue culture reagents contained less than 0.25 ng/mL endotoxin as measured by the limulus amebocyte assay. Murine D10 T cells were obtained from the American Type Culture Collection (Rockville, Md.).

Aortic smooth muscle cells were cultured by explanting segments of the medial layer of aortas from adult male Fischer 344 rats. Aortas were removed aseptically and freed of adventitial and endothelial cells by scraping both the luminal and abluminal surfaces. Medial fragments were allowed to attach to Primaria 25-cm^2 tissue culture flasks (Becton-Dickinson, Lincoln Park, N.J.) which were kept moist with growth medium until cells emerged. Cultures were fed twice weekly with medium 199 containing 10% fetal bovine serum, 25 mM HEPES buffer (pH 7.4), 2mM L-glutamine, 40 μg/mL endothelial cell growth supplement (Biomedical Technologies, Inc., Stoughton, MA) and 10 μg/ml gentamicin (GIBCO BRL, Grand Island, NY). When primary cultures became confluent, they were passaged by trypsinization, and explants were discarded. For these studies, cells from passages 12-14 were seeded at 20,000 per well in 96-well plates and were used at confluence (60,000-80,000 cells per well). The cell exhibited the classic smooth muscle cell phenotype with hill and valley morphology, and they stained positively for smooth muscle actin.

Cell Respiration Assay

Rat aortic smooth muscle cells in 96-well microtiter plates were incubated for 90 minutes in RPMI-1640 medium containing 0.2 mg/mL 3-(4,5 dimethylthiazol-2-γl)-2,5-diphenyltetrazolium bromide (MTT), washed with Hanks' balanced salt solution, and solubilized in 100 μL of dimethyl sulfoxide. The extent of reduction of MTT to formazan within cells, quantitated by measurement of the optical density at 550 nm (OD_{550}), was taken as an

indicator of cellular respiration (Klostergaard, J., *et al*. J. Immunol. Methods 101:97-108, 1987).

IL-1-Induced Cell Proliferation Assay

Murine D10 cells, an IL-1 dependent T-cell line, were used to measure IL-1 mitogenic activity. Cell proliferation in the present of IL-1 was assessed by incorporation of (^{3}H) thymidine as previously described (Bakouche, O., *et al*. J. Immunol. 138:4249-4255, 1987).

IL-1-Induced Cytotoxicity Assay

IL-1-induced cytotoxicity was studied using A375 tumor cells plated at a density of 6000 cells per well in 96-well microliter plates. After overnight attachment, IL-1 (3-300 ng/mL) was added in the presence or absence of NAA or NMA. After cells were incubated for 3 days, (^{3}H) thymidine was added (1 μCi per well) for an additional 2 hours. Cells were harvested onto glass fiber disks (PHD Cell Harvested; Cambridge Technology, Inc., Watertown, Ma.) Disks were air dried overnight, and radioactivity was determined with a Model 1900TR Scintillation Counter (Packard Instrument Division, Downers Grove, Il.)

Induction and Assay of Nitrite Synthesis in Smooth Muscle Cells

Rat aortic smooth muscle cells were incubated with RPMI-1640 medium containing 10% bovine calf serum, 25 mM HEPES buffer (pH 7.4), 2mM glutamine, 80 U/mL penicillin, 80 μg/mL streptomycin, 2 μg/mL fungizone, and IL-1, IFN-γ, and various inhibitors at the concentrations indicated in the figure legends. At the desired times, nitrite concentration in the culture medium was measured using the standard Griess assay (Green, L., *et al*. Anal. Biochem. 126:131-138, 1982) adapted to a 96-well microtiter plate reader (Gross, S.S., *et al*. Biochem. Biophys. Res. Commun. 178:823-829, 1991). Thus, 100 μL of Griess reagent (0.5% sulfanilic acid, 0.05%

naphthalenediamine, and 2.5% phosphoric acid) was added to an equal volume of culture medium, and the OD_{550} was measured and related to nitrite concentration by reference to a standard curve. The background OD_{550} of medium incubated in the absence of cells was subtracted from experimental values.

Preparation and Assay of Smooth Muscle Cell NO Synthase

Rat aortic smooth muscle cells were incubated with RPMI-1640 medium containing 10% bovine calf serum, 25 mM HEPES buffer (pH 7.4), 2 mM glutamine, 80 μg/mL penicillin, 80 μg/mL steptomycin, 2 μg/mL fungizone, 30 μg/mL lipopolysaccharide (*Escherichia coli* 0111:B4), and 50 U/mL IFN-γ. Cells were harvested after 24 hours, and cytosol was prepared (Gross, S.S., *et al.* Biochem. Biophys. Res. Commun. 178:823-829, 1991). Cytosolic NO synthase activity was assayed by the Fe^{2+} - myoglobin method described previously (Gross, S.S., *et al.* Biochem. Biophys. Res. Commun. 178:823-829, 1991).

RESULTS

Induction of Nitric Oxide Synthase in Vascular Smooth Muscle Cells

When grown in the absence of biological response modifiers, rat aortic smooth muscle cells showed no evidence of nitric oxide synthase activity. When cultured in the presence of human recombinant IL-1 (40 ng/mL), however, these cells formed and released substantial amounts of nitrite, a stable degradation product of NO. Nitrite synthesis was evident within 14 hours and continued to increase for at least 40 hours (Fig. 28). These observations are in accord with recent reports that IL-1 induces rat (Beasley, D., *et al.* J. Clin. Invest. 87:602-608, 1991) and rabbit (Busse & Mulsch Febs Lett. 275:87-90, 1990) aortic smooth muscle cells in culture to release a factor that activates guanylate cyclase and decays to nitrite. As shown in

Fig. 28, IFN-γ, which does not itself induce nitric oxide synthase in smooth muscle cells, significantly enhanced the induction of synthase by IL-1. IL-1 receptor antagonist inhibited the induction of nitric oxide synthase by IL-1 or IL-1 plus IFN-γ by more than 98%. Concentration levels of IL-1 as low as 1 ng/mL induced detectable nitric oxide synthase activity in smooth muscle cells; the ED_{50} (i.e., dose which gives a response that is 50% of maximum) values for IL-1 alone and for IL-1 plus IFN-γ were about 5 ng/mL and 1 ng/mL, respectively (Fig. 28, Panel B).

Induction of nitric oxide synthase was dependent on both RNA and protein synthesis. Thus, control monolayers of smooth muscle cells cultured for 14 hours in the presence of IL-1 (100 ng/mL) and IFN-γ (50 ng/mL) produced 2.52 = 0.28 mnol (mean ± SD) of nitrite when transferred to cytokine-free medium and cultured for an additional 16 hours. Equal numbers of smooth muscle cells cultured similarly in medium supplemented with either 0.5 mg/mL of dactinomycin or 1 mg/mL of cycloheximide produced less than 0.1 nmol of nitrite. Neither dactinomycin nor cycloheximide adversely affected cell viability under these conditions, as judged by reduction of MTT, a measure of mitochondrial respiration. (MTT reduction [mean ± SD] by dactinomycin- and cycloheximide-treated IL-1-activated cells was 101% ± 6% and 95% ± 7%, respectively, of that measured in control cells that were treated with IL-1 alone.)

Those of skill in the pharmaceutical and/or neurophysiological arts will be able to practice the present invention with the aid of the disclosure provided here, the following references may facilitate practice or enhanced understanding of certain aspects. Inclusion of a reference in this list is not intended to and does not

constitute an admission that the reference constitutes prior art with respect to the present invention.

The following references are specifically incorporated herein by reference in pertinent part for the purposes indicated herein.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for maintaining an animal's blood pressure at a physiologically acceptable level, the method comprising administering to an animal an arginine-free composition comprising:

alanine;
proline;
serine;
leucine;
methionine;
phenylalanine;
lysine;
valine
isoleucine;
threonine;
tryptophan;
histidine;
tyrosine;
glycine; and
aspartic acid.

2. A method for preventing systemic hypotension induced by tumor necrosis factor or endotoxin in an animal comprising administering to an animal a composition comprising:

less than 1% arginine;
leucine;
methionine;
phenylalanine;
lysine;

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valine;
isoleucine;
threonine;
tryptophan;
alanine;
proline;
serine;
tyrosine;
glycine;
histidine; and
aspartic acid
in a pharmaceutically acceptable diluent.

3. A method for treating hypotension in an animal comprising administering to an animal having hypotension a composition comprising:

0.1-1 g/l arginine;
3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid.

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4. The method of claim 3 wherein the composition is further defined as containing ornithine and citrulline.

5. The method of claim 4 wherein the composition is further defined as containing 1-2 g/l ornithine and 1 g/l citrulline.

6. A method for treating hypotension in an animal, said method comprising administering to an animal with hypotension an arginine-free composition comprising:

1-2 g/l ornithine;
3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid.

together in a pharmaceutically acceptable excipient.

7. A composition comprising an arginine analog and amino acids, wherein the amino acids comprise:

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3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid.

together in a pharmaceutically acceptable excipient.

8. The composition of claim 7 wherein the arginine analog is N^W-amino-L arginine, N^W-methyl-L-arginine, or a mixture thereof.

9. The composition of claim 7, wherein the arginine analog is N^W-methyl-L-arginine.

10. The composition of claim 7 comprising 1-2 g/l ornithine.

11. A method for treating hypotension comprising administering to an animal with hypotension an arginine analog and an arginine-free mixture of amino acids, wherein the amino acids comprise:

3-4 g/l isoleucine;
4-6 g/l leucine;

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3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

in a pharmaceutically acceptable excipient.

12. The method of claim 11 wherein the arginine analog is N^W-amino-L-arginine, N^W-nitro-L-arginine, N^W-methyl-L-arginine, or a mixture thereof.

13. The method of claim 12 wherein the arginine analog is N^W-methyl-L-arginine.

14. The method of claim 11 wherein the arginine analog is N^W-methyl-L-arginine and is administered at a concentration sufficient to provide the animal with between 0.1 mg/kg to 100 mg/kg of the arginine analog.

15. The method of claim 14 wherein the N^W-amino-L-arginine is administered at a concentration sufficient to provide the animal with between 10 mg/kg to 30 mg/kg of the arginine analog.

16. The method of claim 15 wherein the N^W-amino-L-arginine is administered at a concentration sufficient to provide the animal with 10 mg/kg of the arginine

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analog.

17. The method of claim 11 wherein the hypotension is a chemotherapeutic agent related hypotension.

18. The method of claim 17 wherein the chemotherapeutic agent is tumor necrosis factor, interleukin-2, or interleukin-1.

19. The method of claim 15 wherein the N^{W}-methyl-L-arginine is administered at a concentration sufficient to provide the animal with 20 mg/kg of the arginine analog.

20. The method of claim 11 wherein the animal is further defined as having hypotension attendant to septic shock.

21. A method of providing nutritional support to an animal at risk for hypotension comprising administering a nitric oxide synthase inhibitor and an arginine-free mixture of amino acids wherein the arginine-free mixture of amino acids comprises:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;

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0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

22. The method of claim 21 wherein the nitric oxide synthase inhibitor is N^W-methyl-L-arginine, N^W-amino-L-arginine or N^W-nitro-L-arginine, or a mixture thereof.

23. The method of claim 21 wherein the nitric oxide synthase inhibitor is N^W-methyl-L-arginine.

24. A composition comprising an anti-endotoxin antibody and an arginine-free mixture of amino acids wherein the amino acids comprise:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

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together in a pharmaceutically acceptable excipient.

25. The composition of claim 24 further defined as a parenteral or enteral composition.

26. The composition of claim 24 comprising 1-2 g/l ornithine.

27. The composition of claim 25 comprising 1-2 g/l citrulline.

28. The composition of claim 24 wherein the anti-endotoxin antibody is HA-1A.

29. A composition comprising an interleukin-1 receptor antagonist and amino acids wherein the amino acids are further defined as:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

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30. The composition of claim 29 comprising 1-2 g/l ornithine.

31. The composition of claim 29 comprising 1-2 g/l citrulline.

32. The composition of claim 29 wherein the interleukin-1 receptor antagonist is IL 1ra.

33. A composition comprising an anti-tumor necrosis factor antibody and amino acids wherein the amino acids comprise:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

34. The composition of claim 33 wherein the anti-tumor necrosis factor antibody is a monoclonal antibody or a polyclonal antibody.

35. The composition of claim 34 wherein the anti-tumor necrosis factor antibody

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is a monoclonal antibody.

36. The composition of claim 35 wherein the anti-tumor necrosis factor monoclonal antibody is CB0006.

37. The composition of claim 33 further comprising 1-2 g/l ornithine.

38. The composition of claim 33 further comprising 1-2 g/l citrulline.

39. A method for treating hypotension comprising administering to an animal having hypotension an arginine-free mixture of amino acids, and either an anti-tumor necrosis factor antibody or an interleukin-1 receptor antagonist, wherein the arginine-free mixture of amino acids comprises:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient and wherein said antibody or antagonist is administered concurrently with or after the arginine-free mixture of

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amino acids.

40. The method of claim 39 wherein the anti-tumor necrosis factor antibody is a monoclonal antibody or a polyclonal antibody.

41. The method of claim 40 wherein the antibody is a monoclonal antibody.

42. The method of claim 41 wherein the monoclonal antibody is CB0006.

43. The method of claim 39 wherein between 0.1 mg/kg to 20 mg/kg of the anti-tumor necrosis factor antibody is administered to the animal.

44. The method of claim 39 wherein the interleukin-1 receptor antagonist is administered to the animal in an amount of between 1 mg/kg to 100 mg/kg.

45. The method of claim 39 wherein the arginine-free mixture of amino acids is further defined as a parenteral composition.

46. The method of claim 39 wherein the anti-tumor necrosis factor antibody is administered to the animal in an amount of 0.1 mg/kg to 20 mg/kg.

47. The method of claim 46 wherein the anti-tumor necrosis factor antibody is CB0006.

48. A composition comprising an arginine-free mixture of amino acids and an anti-endotoxin antibody wherein the mixture of amino acids comprises:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;

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2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

49. The composition of claim 48 wherein the anti-endotoxin antibody is HA-1A.

50. A composition comprising an arginine-free mixture of amino acids and either an interleukin-1 receptor antagonist or an anti-tumor necrosis factor antibody wherein the arginine-free mixture of amino acids comprise:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;

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4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient.

51. The composition of claim 50 wherein the interleukin-1 receptor antagonist is IL 1ra.

52. A method for treating septic shock related hypotension in an animal exposed to endotoxin comprising administering an arginine-free mixture of amino acids and either an interleukin-1 receptor antagonist or an anti-endotoxin antibody wherein the amino acids comprise:

3-4 g/l isoleucine;
4-6 g/l leucine;
3-4 g/l lysine;
1-2 g/l methionine;
1-2 g/l phenylalanine;
2-3 g/l threonine;
0.5-1.5 g/l tryptophan;
3-4 g/l valine;
4-5 g/l alanine;
1-2 g/l histidine;
3-4 g/l proline;
1-2 g/l serine;
0.25-0.75 g/l tyrosine;
4-5 g/l glycine; and
2-3 g/l aspartic acid,

together in a pharmaceutically acceptable excipient and wherein said antagonist or antibody is administered concurrently with or after the arginine-free mixture of amino acids.

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53. The method of claim 52 wherein the interleukin-1 receptor antagonist is IL 1ra and the anti-endotoxin antibody is HA-1A.

Dated this 12th day of September, 1995

Board of Regents, The University of Texas System AND Cornell Research Foundation, Inc.

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicant(s)

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FIG.1

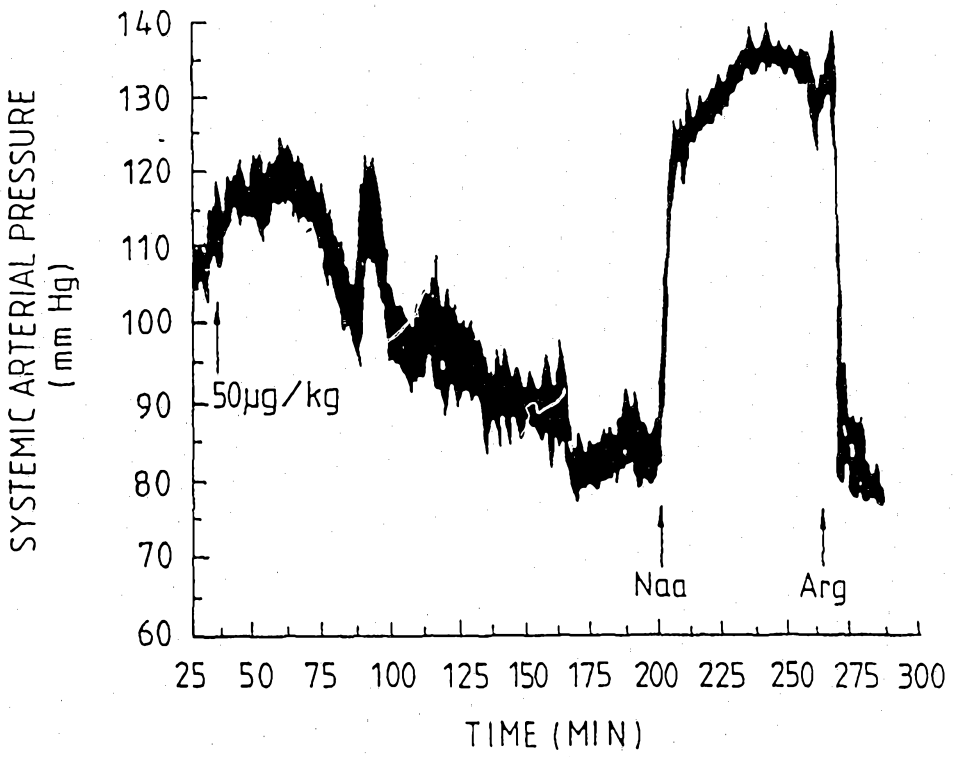

SYSTEMIC ARTERIAL PRESSURE
(mm Hg)
140
130
120
110
100
90
80
70
60
50µg/kg
Naa
Arg
25 50 75 100 125 150 175 200 225 250 275 300
TIME (MIN)

FIG.2A

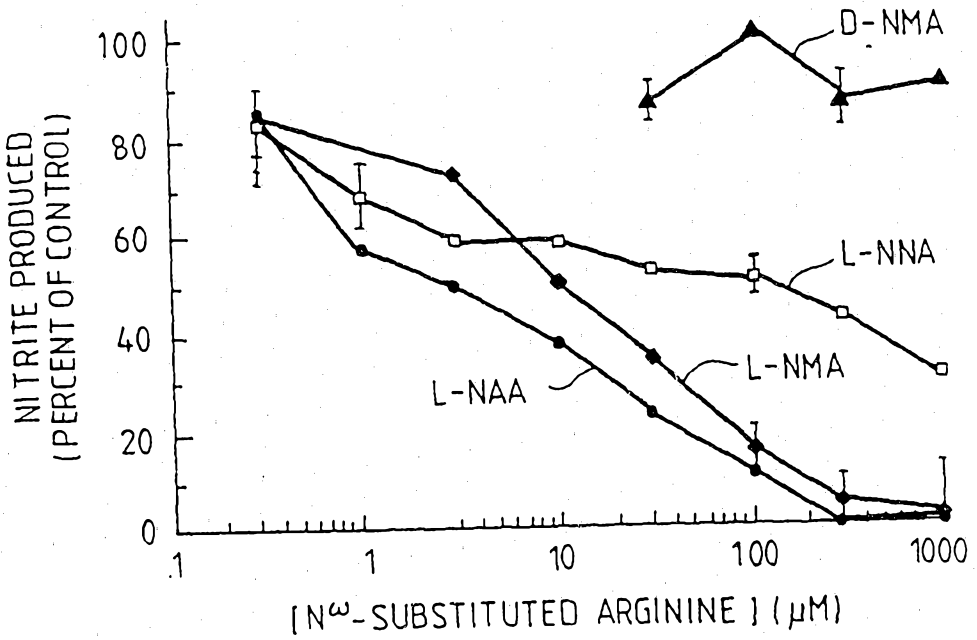

D-NMA
L-NNA
L-NMA
L-NAA
NITRITE PRODUCED
(PERCENT OF CONTROL)
100
80
60
40
20
0
.1
1
10
100
1000
[Nω-SUBSTITUTED ARGININE] (μM)

FIG.2B

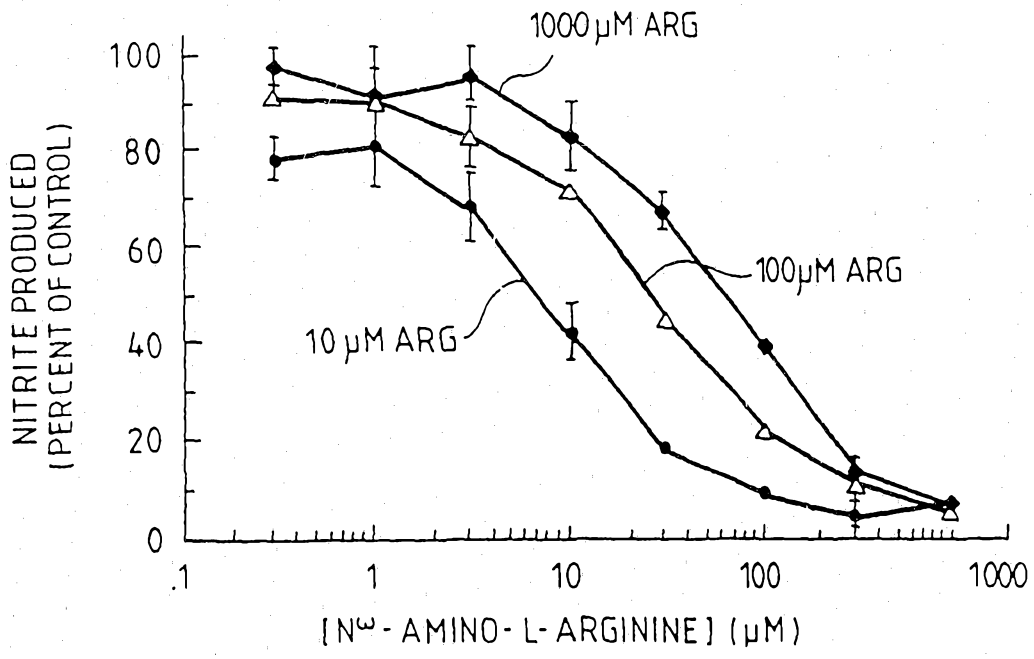

1000 μM ARG
100μM ARG
10 μM ARG
NITRITE PRODUCED
(PERCENT OF CONTROL)
100
80
60
40
20
0
.1
1
10
100
1000
[Nω - AMINO - L - ARGININE] (μM)

FIG.2C

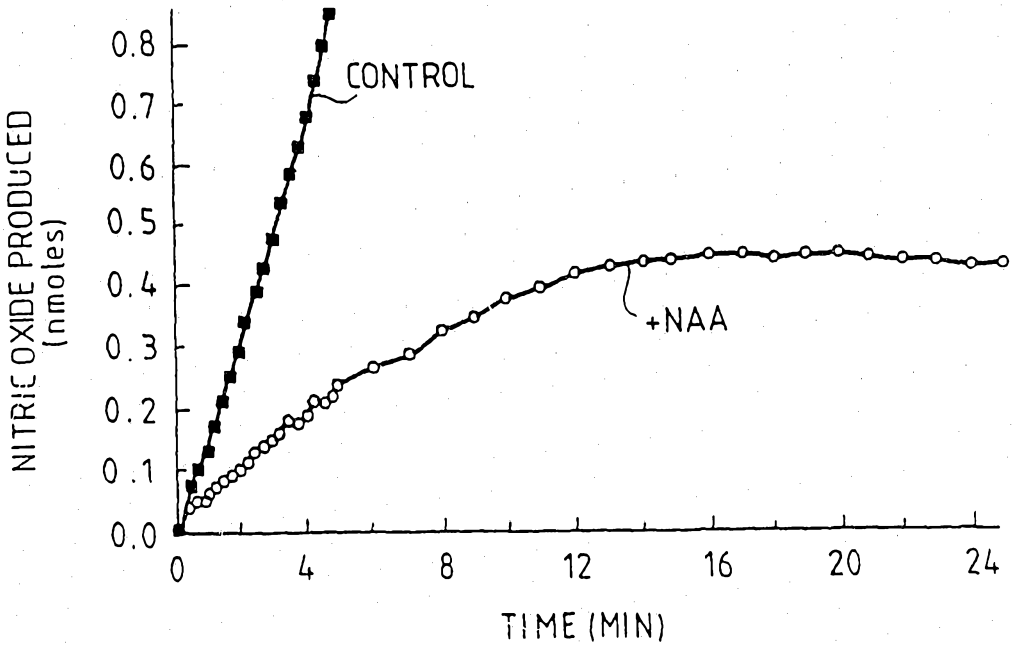

CONTROL
+NAA
NITRIC OXIDE PRODUCED
(nmoles)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
20
24
TIME (MIN)

FIG.3

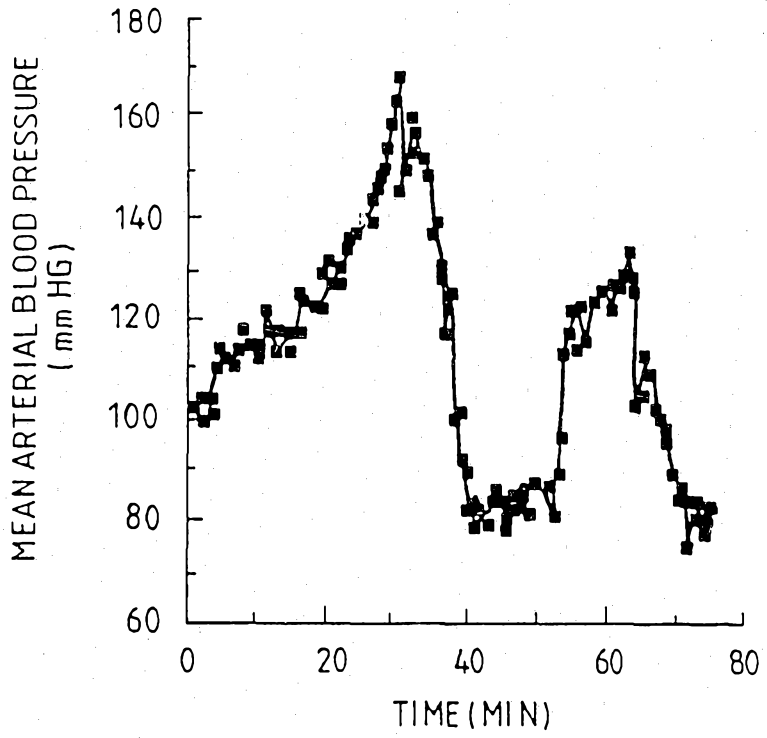

MEAN ARTERIAL BLOOD PRESSURE
(mm HG)
180
160
140
120
100
80
60
0
20
40
60
80
TIME (MIN)

FIG.4A

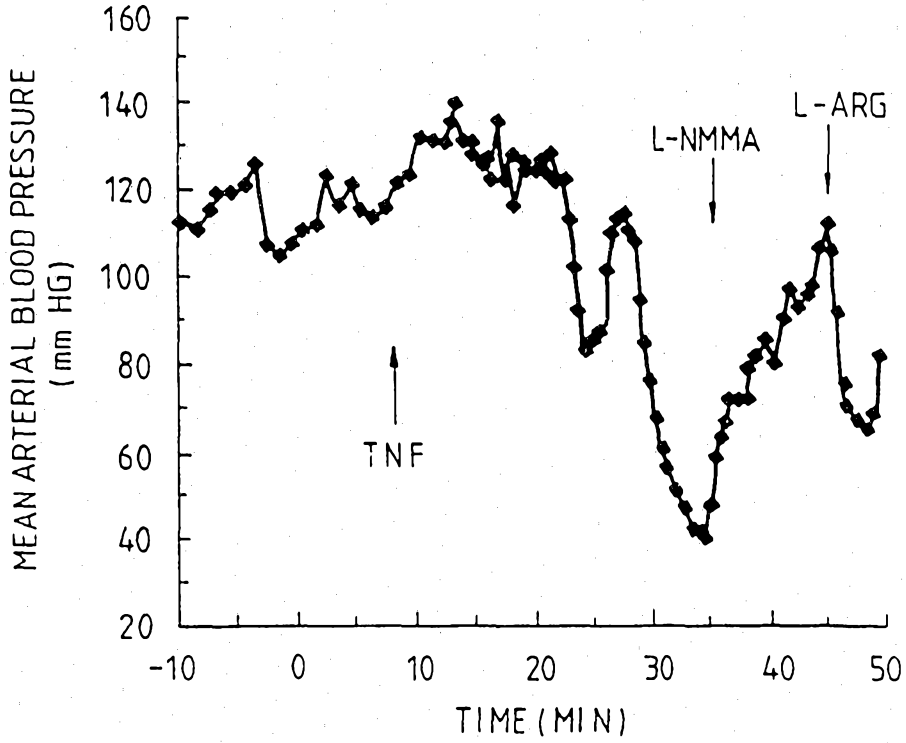

MEAN ARTERIAL BLOOD PRESSURE
(mm HG)
160
140
120
100
80
60
40
20
-10
0
10
20
30
40
50
TIME (MIN)
TNF
L-NMMA
L-ARG

FIG. 4B

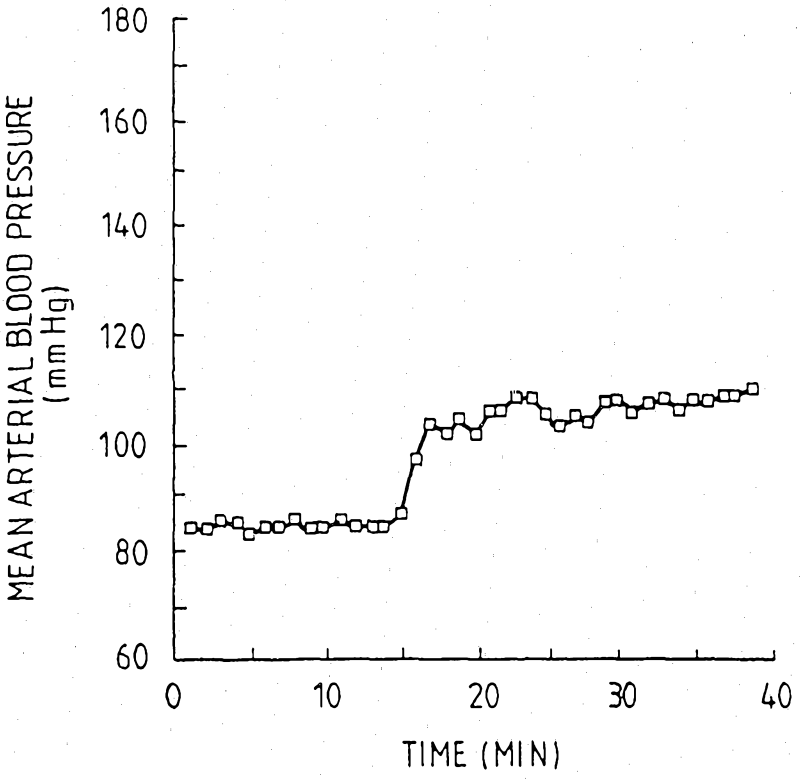

MEAN ARTERIAL BLOOD PRESSURE
(mm Hg)
180
160
140
120
100
80
60
0
10
20
30
40
TIME (MIN)

FIG. 4C

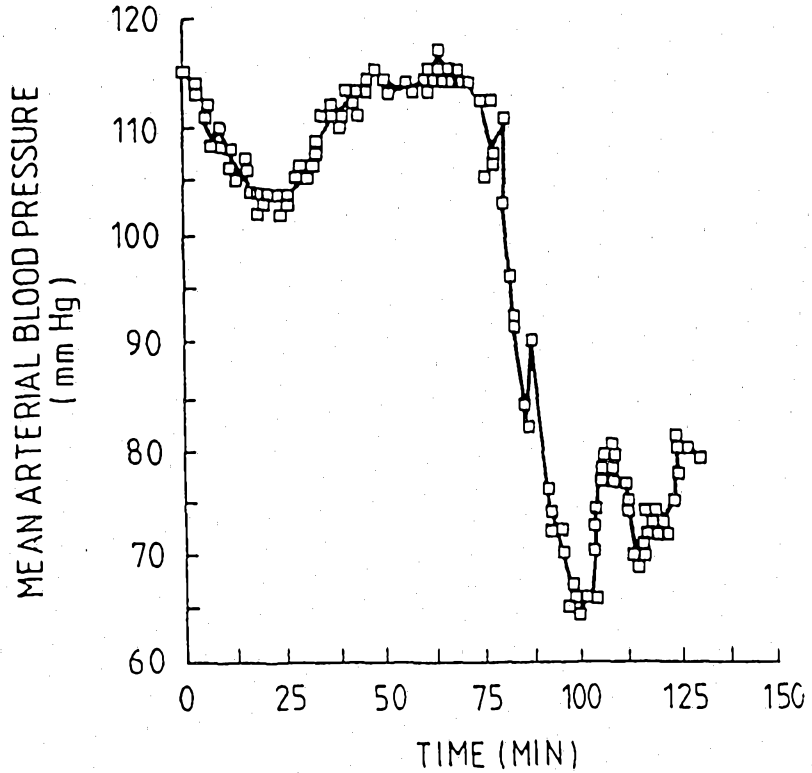

MEAN ARTERIAL BLOOD PRESSURE
(mm Hg)
120
110
100
90
80
70
60
0
25
50
75
100
125
150
TIME (MIN)

FIG.5

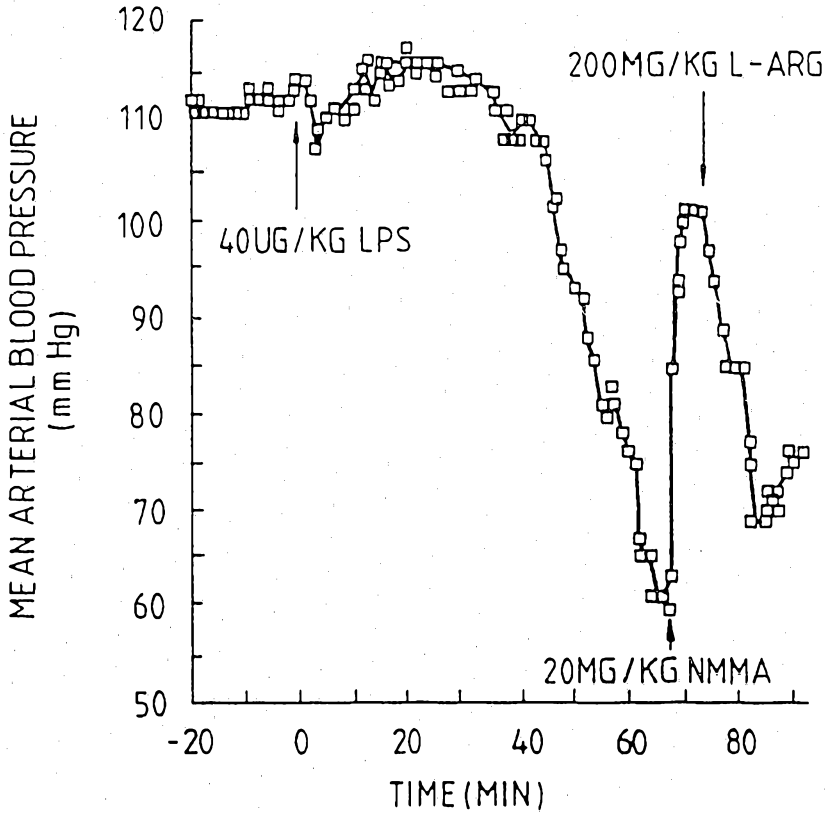
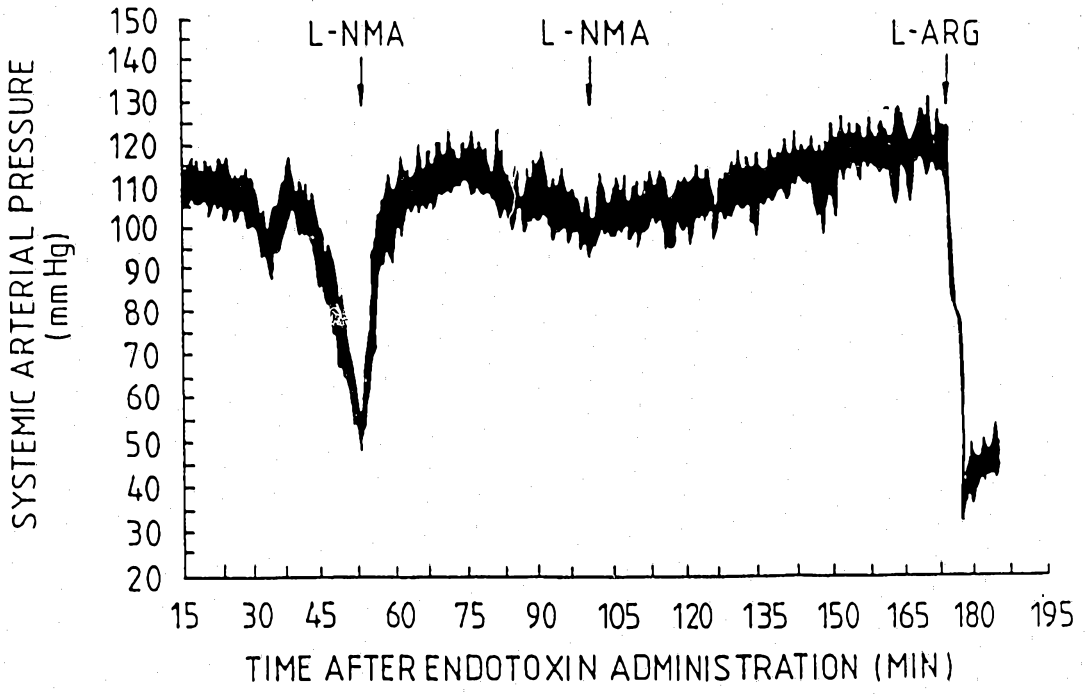

MEAN ARTERIAL BLOOD PRESSURE (mm Hg)
120
110
100
90
80
70
60
50
200MG/KG L-ARG
40UG/KG LPS
20MG/KG NMMA
-20
0
20
40
60
80
TIME (MIN)

FIG.6


SYSTEMIC ARTERIAL PRESSURE (mmHg)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
L-NMA
L-NMA
L-ARG
15
30
45
60
75
90
105
120
135
150
165
180
195
TIME AFTER ENDOTOXIN ADMINISTRATION (MIN)

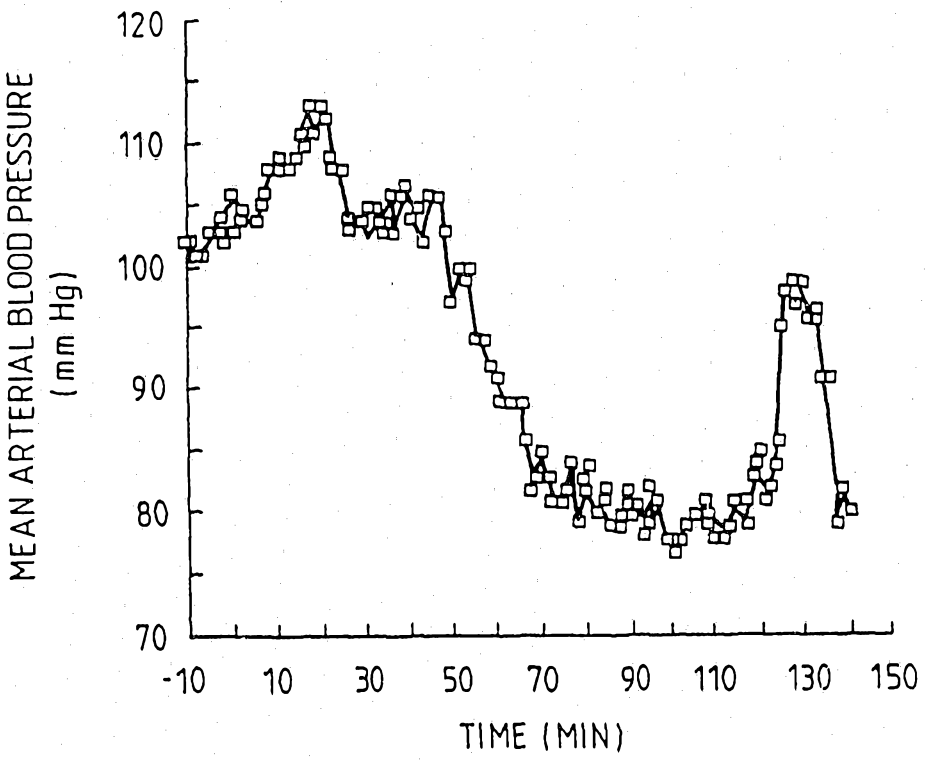
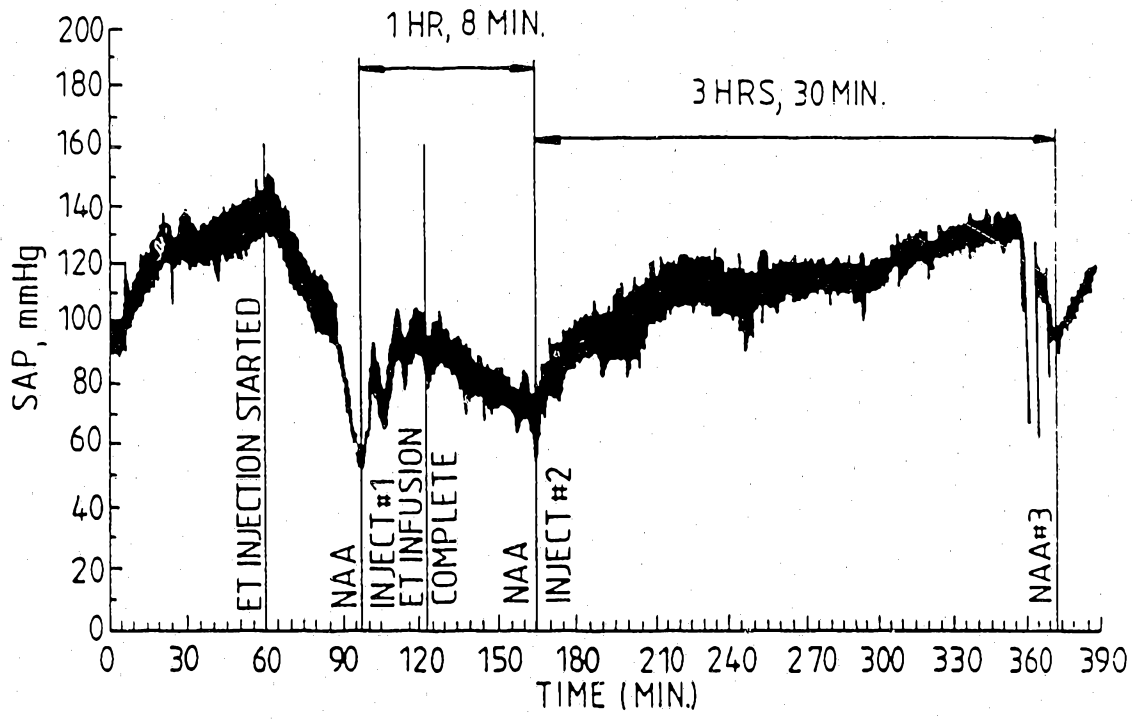

MEAN ARTERIAL BLOOD PRESSURE
(mm Hg)
120
110
100
90
80
70
-10
10
30
50
70
90
110
130
150
TIME (MIN)

FIG.7

FIG.8


1 HR, 8 MIN.
3 HRS, 30 MIN.
SAP, mmHg
200
180
160
140
120
100
80
60
40
20
0
ET INJECTION STARTED
NAA
INJECT #1
ET INFUSION
COMPLETE
NAA
INJECT #2
NAA#3
0 30 60 90 120 150 180 210 240 270 300 330 360 390
TIME (MIN.)

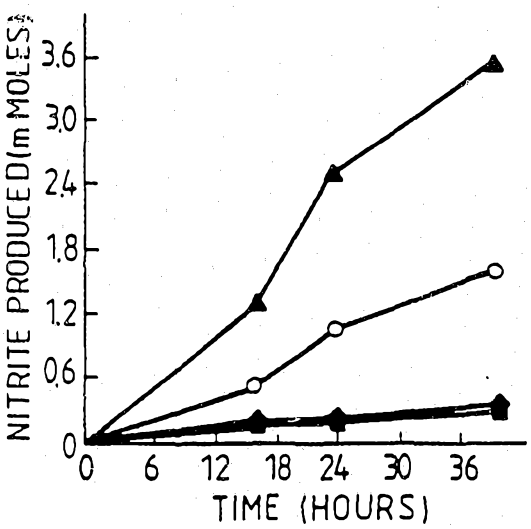

NITRITE PRODUCED (m MOLES)
3.6
3.0
2.4
1.8
1.2
0.6
0
0 6 12 18 24 30 36
TIME (HOURS)

FIG.9A

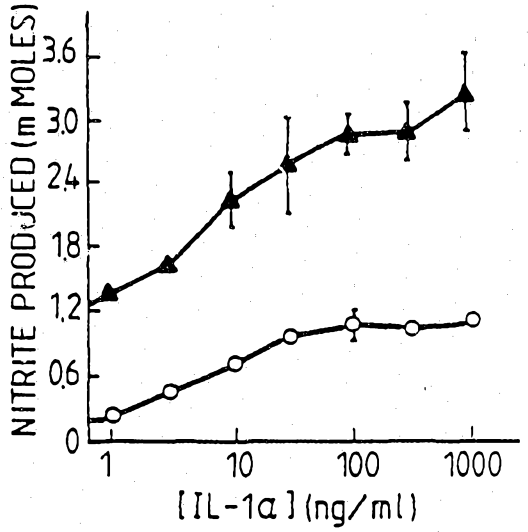

NITRITE PRODUCED (m MOLES)
3.6
3.0
2.4
1.8
1.2
0.6
0
1 10 100 1000
[IL-1α] (ng/ml)

FIG.9B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/08227

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 A61K31/415; A61K37/02; //(A61K37/02,31:415,31:405,31:40 ,31:195)

II. FIELDS SEARCHED	
Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	A61K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]	

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	DE,A,2 516 027 (THE JOHNS HOPKINS UNIVERSITY) 16 October 1975 see claims	1-88
A	US,A,5 006 559 (JEFFREY ASKANAZI & KAREN GIL) 9 April 1991 see claims	1-88
A	EP,A,0 318 446 (AB ERIC VINNARS) 31 May 1989 see claim 1	1-88

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 JANUARY 1993	10. 02. 93
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LEHERTE C.F.M.

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 92/ 08227

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
"Remark : Although claims 12-88 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition."

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9208227
SA 65659

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 18/01/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-2516027	16-10-75	BE-A- 827890	31-07-75
		CA-A- 1057198	26-06-79
		FR-A,B 2274290	09-01-76
		GB-A- 1511302	17-05-78
		NL-A- 7504408	17-10-75
		US-A- 4100160	11-07-78
US-A-5006559	09-04-91	None	
EP-A-0318446	31-05-89	AU-A- 2623888	23-05-89
		DE-A- 3875322	19-11-92
		EP-A- 0398879	28-11-90
		JP-T- 3500775	21-02-91
		WO-A- 8903688	05-05-89