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(54) Title: METHODS OF TREATING NECROTIZING ENTEROCOLITIS

(57) Abstract: The present invention relates to a method of treating necrotizing enterocolitis in a patient, which includes administering a pharmaceutical composition that includes carbon monoxide to the patient.

METHODS OF TREATING NECROTIZING ENTEROCOLITIS

Statement as to Federally Sponsored Research

This invention was made with Government support under National Institutes of Health Grant Nos. HL55330, HL60234, and AI42365. The Government has certain rights in this invention.

Technical Field

This invention relates to the treatment of gastrointestinal disorders.

Background

Carbon monoxide (CO) is recognized as an important signaling molecule (Verma *et al.*, Science 259:381-384, 1993). It has also been suggested that CO acts as a neuronal messenger molecule in the brain (*Id.*) and as a neuro-endocrine modulator in the hypothalamus (Pozzoli *et al.*, Endocrinology 735:2314-2317, 1994). Like nitric oxide (NO), CO is a smooth muscle relaxant (Utz *et al.*, Biochem Pharmacol. 47:195-201, 1991; Christodoulides *et al.*, Circulation 97:2306-9, 1995) and inhibits platelet aggregation (Mansouri *et al.*, Thromb Haemost. 48:286-8, 1982). Inhalation of low levels of CO has been shown to have anti-inflammatory effects in some models.

Necrotizing enterocolitis (NEC) is a disease of newborns characterized by gut barrier failure, intestinal necrosis, sepsis, and multi-system organ failure (see, e.g., Oxford Textbook of Surgery, Morris and Malt, Eds., Oxford University Press (1994)).

Summary

The present invention is based, in part, on the discovery that administration of CO can protect against the development of NEC.

Accordingly, the present invention features a method of treating, preventing, or reducing the risk of necrotizing enterocolitis in a patient. The method includes identifying a patient suffering from or at risk for necrotizing enterocolitis and administering to the patient a pharmaceutical composition comprising an amount of CO effective to treat necrotizing enterocolitis in the patient. The method can include the further step of monitoring the patient's NEC condition and/or determining whether the patient's condition has improved or the risk of NEC has abated.

The pharmaceutical composition can be administered to the patient by any method known in the art for administering gases, liquids, and/or solids to patients, e.g., via inhalation, insufflation, infusion, injection, and/or ingestion. In one embodiment of the present invention, the pharmaceutical composition is administered to the patient by inhalation. In another embodiment, the pharmaceutical composition is administered to the patient orally. In still another embodiment, the pharmaceutical composition is administered directly to the abdominal cavity of the patient. In yet another embodiment, the pharmaceutical composition is administered by an extracorporeal membrane gas exchange device or an artificial lung.

The patient can be an infant, e.g., a full-term infant, a premature infant and/or an infant that exhibits low birth weight. The infant can be a newborn, or can be, e.g., up to one year old (for example, up to six months, four months, three months, or two months old). The infant can be less than six weeks old, e.g., less than four weeks old. The NEC can be the result of any of a number of factors, e.g., hypoxia, hypothermia, hypotension, hyperviscosity of the blood, and/or acidosis, and/or where the patient has received an exchange transfusion, at least one hyperosmolar feed, a packed cell transfusion, and/or an overdosage of calcium antagonists. Further, the NEC can result from a situation where the patient suffers from mesenteric ischaemia and/or bacterial infection of the bowel wall (see, e.g., *Oxford Textbook of Surgery*, Morris and Malt, Eds., Oxford University Press (1994)). Alternatively, the NEC can result from surgery, e.g., where the patient has undergone, is about to undergo, or is undergoing surgery. The pharmaceutical composition can be in any form, e.g., gaseous or liquid form.

The invention also features a method of treating or preventing necrotizing enterocolitis in a patient, which includes identifying a patient suffering from or at risk for necrotizing enterocolitis, providing a vessel containing a pressurized gas comprising CO gas, releasing the pressurized gas from the vessel to form an atmosphere comprising CO gas, and exposing the patient to the atmosphere, wherein the amount of CO in the atmosphere is sufficient to treat necrotizing enterocolitis in the patient.

In another aspect, the invention features a method of performing abdominal surgery on a patient (e.g., an infant), which includes identifying a patient in need of abdominal surgery, wherein necrotizing enterocolitis is a significant risk of the abdominal surgery; performing abdominal surgery on the patient, and before, during, or

after the performing step, causing the patient to inhale an amount of CO gas sufficient to reduce the risk of necrotizing enterocolitis in the patient.

Also included in the invention is a method of treating necrotizing enterocolitis in a patient, which includes: (a) identifying a patient suffering from necrotizing enterocolitis; (b) performing surgery on the patient to resect an affected portion of the patient's bowel; and (c) administering to the patient a pharmaceutical composition comprising an amount of carbon monoxide effective to treat necrotizing enterocolitis in the patient after step (a) and before, during, or after step (b).

In another aspect, the invention provides a vessel comprising medical grade compressed CO gas. The vessel can bear a label indicating that the gas can be used to treat NEC in a patient, e.g., an infant. The CO gas can be in an admixture with nitrogen gas, with nitric oxide and nitrogen gas, or with an oxygen-containing gas. The CO gas can be present in the admixture at a concentration of at least about 0.025%, e.g., at least about 0.05%, 0.10%, 0.50%, 1.0%, 2.0%, 10%, 50%, or 90%.

In still another aspect, the invention provides a method of treating NEC in a patient, which includes identifying a patient suffering from or at risk for NEC and administering to the patient at least one of the following treatments in conjunction with treatment with CO: inducing HO-1 or ferritin in the patient; expressing recombinant HO-1 or ferritin in the patient; and administering a pharmaceutical composition comprising HO-1, bilirubin, biliverdin, ferritin, or apoferritin, iron, desferoxamine, or iron dextran to the patient. Also contemplated is use of CO and any of the above-listed agents in the preparation of a medicament for treatment or prevention of NEC.

Further, the invention provides a method of treating NEC in a patient, which includes identifying a patient suffering from or at risk for NEC and administering at least one of the following treatments in conjunction with treatment with CO: intravenous nutrition; intravenous hydration; antimicrobial agents; performing nasogastric decompression on the patient, performing surgery on the patient; and draining the patient's peritoneal cavity.

Also within the invention is the use of CO in the manufacture of a medicament for treatment or prevention of NEC. The medicament can be used in a method for treating NEC in a patient suffering from or at risk for NEC in accordance with the

methods described herein. The medicament can be in any form described herein, e.g., a liquid or gaseous CO composition.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Suitable methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the claims.

Description of the Drawings

Fig. 1A is a picture of a Western blot illustrating that HO-1 expression is increased in intestinal samples from human neonates suffering from NEC (NEC) as compared to non-NEC patients (control) (n = 3).

Fig. 1B is a picture of a Western blot illustrating that ileal HO-1 protein is increased at day four in neonatal rats subjected to intermittent hypoxia and formula feeding (NEC) as compared to breast fed control rats (control) (n=4).

Fig. 2A is a photomicrograph (40X magnification) of a hematoxylin and eosin-stained ileal whole mount illustrating the effect of breast feeding on a neonatal rat. The sample was obtained on day four.

Fig. 2B is a photomicrograph (40X magnification) of a hematoxylin and eosin-stained ileal whole mount illustrating the effect of breast feeding and CO exposure on a neonatal rat. The sample was obtained on day four.

Fig. 2C is a photomicrograph (40X magnification) of a hematoxylin and eosin-stained ileal whole mount illustrating the effect of formula feeding plus hypoxia exposure on a neonatal rat. The sample was obtained on day four. Architectural changes including villous atrophy and cellular vacuolization can be observed.

Fig. 2D is a photomicrograph (40X magnification) of a hematoxylin and eosin-stained ileal whole mount illustrating the effect of formula feeding, plus hypoxia exposure and CO exposure on a neonatal rat. Fewer architectural changes occur as compared to the sample shown in Fig. 2C. The sample was obtained on day four.

Fig. 3A is a photomicrograph (60X magnification) of a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)-stained ileal whole mount illustrating the effect of breast feeding on a neonatal rat. The sample was obtained on day four.

Fig. 3B is a photomicrograph (60X magnification) of a TUNEL-stained ileal whole mount illustrating the effect of breast feeding and CO exposure on a neonatal rat. The sample was obtained on day four.

Fig. 3C is a photomicrograph (60X magnification) of a TUNEL-stained ileal whole mount illustrating the effect of formula feeding plus hypoxia exposure on a neonatal rat. The sample was obtained on day four. Ileum from hypoxia/formula-fed treated rats exhibits increased TUNEL staining compared to that from breast fed animals.

Fig. 3D is a photomicrograph (60X magnification) of a TUNEL-stained ileal whole mount illustrating the effect of formula feeding plus hypoxia exposure, and CO exposure on a neonatal rat. The sample was obtained on day four. A decrease in TUNEL positive cells can be observed.

Fig. 4A is a bar graph illustrating that CO treatment prevents an increase in the serum IL-1β level in hypoxia-exposed plus formula-fed neonatal rats as compared to controls ($P<0.05$). The data was generated using an ELISA assay. Black bars = air-exposed rats; gray bars = CO exposed rats.

Fig. 4B is a bar graph illustrating that CO treatment prevents an increase in the serum TNF-α level in hypoxia-exposed and formula-fed neonatal rats as compared to controls ($P<0.05$). The data was generated using an ELISA assay. Black bars = air-exposed rats; gray bars = CO-exposed rats.

Fig. 5 is a picture of a Western blot illustrating that CO treatment decreases ileal expression of COX-2 and IL-1β in hypoxia-exposed and formula-fed neonatal rats, as compared to controls. The presence (+) or absence (-) of each treatment (breast feeding (BF), formula-feeding plus hypoxia exposure (FF/Hypoxia) and CO exposure (CO)) is

indicated beneath each lane of the Western blot. Blot demonstrates 3-4 animals per group and is representative of all animals in the study.

Fig. 6 is a picture of a Western blot illustrating that exposure to CO abrogates experimental NEC-induced ileal iNOS expression and protein nitration in neonatal rats. The presence (+) or absence (-) of each treatment (breast feeding (BF), formula-feeding plus hypoxia exposure (FF/Hypoxia) and CO exposure (CO) is indicated beneath each lane of the Western blot.

Fig. 7 is a bar graph illustrating that CO exposure and induction of HO-1 decreases TNF-α/Actinomycin D (TNF/ActD)-induced IEC-6 cell death. Viability of TNF-α (TNF; 10ng/ml)/Actinomycin D (ActD; 200ng/ml)-treated IEC-6 cells was assayed after 18 hours by measuring cellular ATP content. CO treatment (CO; gray bars; 250 ppm) was initiated 1 hour prior to administration of TNF-α/ActD and maintained throughout the duration of the experiment. Cross-hatched bars = cells exposed to cobalt protoporphyrin (CoPP) 16 hours prior to exposure to TNF-α/ActD. Black bars = air exposed cells. Both CO and CoPP significantly decreased TNF-α/ActD-induced IEC-6 cell death ($P<0.05$). Results are mean ± standard error of 3 independent studies performed in triplicate.

Fig. 8A is a picture of a Western blot illustrating (at 24 hours) that iNOS protein expression is inhibited in lipopolysaccharide (LPS) and/or hypoxia (1% oxygen) treated IEC-6 cells. CO treatment (250ppm) was initiated 1hour before the addition of LPS/hypoxia and maintained throughout the experiment. The combination of LPS (10 or 100 ng/ml) plus hypoxia increased iNOS protein expression, an effect that was inhibited by CO. The presence (+) or absence (-) of each treatment (lipopolysaccharide (lps), hypoxia exposure (hypoxia) and CO exposure (CO)) is indicated beneath each lane of the Western blot.

Fig. 8B is a bar graph illustrating that rat iNOS promoter activity in LPS (100 ng/ml)/hypoxia (1% oxygen; lps/hypoxia)-treated IEC-6 cells is limited by exposure to CO. Cells were assayed for luciferase activity. The combination of LPS plus hypoxia resulted in a 4.9 ± 0.3 fold increase in transcriptional activation of the iNOS promoter ($P<0.05$). CO limited this transcriptional activation to a 1.7 ± 0.2 fold increase ($P<0.05$). Results are mean ± standard error of 3 independent studies performed in triplicate.

Fig. 8C is a picture of a Western blot illustrating that cytokine-induced iNOS protein expression is inhibited in IEC-6 cells by induction of HO-1 or treatment with CO. IEC-6 cells were treated with a cytokine mixture (CM) containing TNF-α (10ng/ml), IL-1β (500 U/ml), and IFN-γ (1000 U/ml) for 24 hours. CO treatment (250 ppm) was initiated 1 hour prior to administration of CM and maintained throughout the duration of the experiment. Cobalt protoporphyrin (CoPP) was administered 16 hours prior to CM treatment. CM increased IEC-6 cell iNOS protein. Both CO and CoPP inhibited cytokine-induced increase in iNOS protein. The presence (+) or absence (-) of each treatment (cytokine mixture (CM), CO exposure (CO), and cobalt protoporphyrin exposure (CoPP)) is indicated beneath each lane of the Western blot.

Fig. 8D is a bar graph illustrating that the nitrite levels in supernatantants of IEC-6 cells that are exposed to CM and either CO or CoPP are lower than those of IEC-6 cells exposed to CM and air (as determined by Griess assay). Cytokine stimulation increased nitrite to 17.2±0.9 μM compared to 1.4 ± 0.3 μM in unstimulated controls ($P<0.01$). CO and CoPP significantly inhibited this cytokine effect resulting in nitrite levels of 9.8 ± 0.7 and 10.4 ± 1.0, respectively ($P<0.05$ compared to CM-stimulated cells). Black bars = air exposed cells; gray bars = CO exposed cells; and cross-hatched bars = CoPP exposed cells.

Detailed Description

The term "carbon monoxide" (or "CO") as used herein describes molecular CO in its gaseous state, compressed into liquid form, or dissolved in aqueous solution. The terms "carbon monoxide composition" and "pharmaceutical composition comprising carbon monoxide" is used throughout the specification to describe a gaseous or liquid composition containing CO that can be administered to a patient and/or an organ, e.g., an organ affected by NEC. The skilled practitioner will recognize which form of the pharmaceutical composition, e.g., gaseous, liquid, or both gaseous and liquid forms, is preferred for a given application.

The terms "effective amount" and "effective to treat," as used herein, refer to an amount or concentration of CO utilized for period of time (including acute or chronic administration and periodic or continuous administration) that is effective within the context of its administration for causing an intended effect or physiological outcome in a patient. Effective amounts of CO for use in the present invention include,

for example, amounts that reduce the symptoms of NEC in a patient, or improve the outcome.

For gases, effective amounts of CO generally fall within the range of about 0.0000001% to about 0.3% by weight, e.g., 0.0001% to about 0.25% by weight, preferably at least about 0.001%, e.g., at least 0.005%, 0.010%, 0.02%, 0.025%, 0.03%, 0.04%, 0.05%, 0.06%, 0.08%, 0.10%, 0.15%, 0.20%, 0.22%, or 0.24% by weight of CO. For liquid solutions of CO, effective amounts generally fall within the range of about 0.0001 to about 0.0044 g CO/100 g liquid, e.g., at least 0.0001, 0.0002, 0.0004, 0.0006, 0.0008, 0.0010, 0.0013, 0.0014, 0.0015, 0.0016, 0.0018, 0.0020, 0.0021, 0.0022, 0.0024, 0.0026, 0.0028, 0.0030, 0.0032, 0.0035, 0.0037, 0.0040, or 0.0042 g CO/100 g aqueous solution. Preferred ranges include, e.g., about 0.0010 to about 0.0030 g CO/100 g liquid, about 0.0015 to about 0.0026 g CO/100 g liquid, or about 0.0018 to about 0.0024 g CO/100 g liquid. A skilled practitioner will appreciate that amounts outside of these ranges may be used, depending upon the application.

The term "patient" is used throughout the specification to describe an animal, human or non-human, to whom treatment according to the methods of the present invention is provided. Veterinary applications are clearly contemplated by the present invention. The term includes but is not limited to mammals, e.g., humans, other primates, pigs, rodents such as mice and rats, rabbits, guinea pigs, hamsters, cows, horses, cats, dogs, sheep and goats. The term "treat(ment)," is used herein to describe delaying the onset of, inhibiting, or alleviating the effects of a condition, e.g., NEC, in a patient.

The term "necrotizing enterocolitis" or "NEC" is an art-recognized term and is used herein to refer to a disease of patients, particularly premature and newborn full term infants, that is characterized by gut barrier failure, intestinal necrosis, sepsis, and multi-system organ failure (*Oxford Textbook of Surgery*, Morris and Malt, Eds., Oxford University Press (1994)). NEC can affect any part of the bowel, e.g., the lower portion of the small intestine (ileum), the colon, and/or the upper small intestine. The risk of developing necrotizing enterocolitis is associated with many factors, e.g., low birth weight, hypoxia, hypothermia, hypotension, hyperviscosity, acidosis, or the presence of free oxygen radicals (*Id.*). Other risk factors include umbilical artery cannulae, exchange transfusion, hyperosmolar feeds, packed cell transfusion, or overdosage with calcium antagonists (*Id.*). Such factors may cause mesenteric ischaemia, which may

allow bacterial infection of the bowel wall, resulting in necrosis of the infected tissue and/or perforation of the bowel wall and septicemia (*Id.*). NEC can also occur in newborns following surgery for gastrointestinal or other conditions (*Id.*).

Skilled practitioners will appreciate that a patient can be diagnosed as suffering from NEC by any method known in the art, e.g., by a physician's diagnosis (e.g., using imaging techniques such as ultrasonography, x-ray, and/or blood tests).

Individuals considered at risk for developing NEC may benefit particularly from the invention, primarily because prophylactic treatment can begin before there is any evidence of NEC. Individuals "at risk" include, e.g., premature and newborn infants, or individuals suffering from any of the conditions or having the risk factors described above. Skilled practitioners will appreciate that a patient can be diagnosed as being at risk for NEC by any method known in the art, e.g., by a physician's diagnosis (e.g., by a physician's assessment of a patient's risk factors).

Preparation of Gaseous Compositions

A CO composition may be a gaseous CO composition. Compressed or pressurized gas useful in the methods of the invention can be obtained from any commercial source, and in any type of vessel appropriate for storing compressed gas. For example, compressed or pressurized gases can be obtained from any source that supplies compressed gases, such as oxygen, for medical use. The term "medical grade" gas, as used herein, refers to gas suitable for administration to patients as defined herein. The pressurized gas including CO used in the methods of the present invention can be provided such that all gases of the desired final composition (e.g., CO, He, NO, CO_2, O_2, N_2) are in the same vessel, except that NO and O_2 cannot be stored together. Optionally, the methods of the present invention can be performed using multiple vessels containing individual gases. For example, a single vessel can be provided that contains CO, with or without other gases, the contents of which can be optionally mixed with room air or with the contents of other vessels, e.g., vessels containing oxygen, nitrogen, carbon dioxide, compressed air, or any other suitable gas or mixtures thereof.

Gaseous compositions administered to a patient according to the present invention typically contain 0% to about 79% by weight nitrogen, about 21% to about 100% by weight oxygen and about 0.0000001% to about 0.3% by weight

(corresponding to about 1 ppb or 0.001 ppm to about 3,000 ppm) CO. Preferably, the amount of nitrogen in the gaseous composition is about 79% by weight, the amount of oxygen is about 21% by weight and the amount of CO is about 0.0001% to about 0.25% by weight. The amount of CO is preferably at least about 0.001%, e.g., at least about 0.005%, 0.010%, 0.02%, 0.025%, 0.03%, 0.04%, 0.05%, 0.06%, 0.08%, 0.10%, 0.15%, 0.20%, 0.22%, or 0.24% by weight. Preferred ranges include about 0.005% to about 0.24%, about 0.01% to about 0.22%, about 0.015% to about 0.20%, about 0.08% to about 0.20%, and about 0.025% to about 0.1% by weight. It is noted that gaseous CO compositions having concentrations of CO greater than 0.3% (such as 1% or greater) may be used for short periods (e.g., one or a few breaths), depending upon the application.

A gaseous CO composition may be used to create an atmosphere that comprises CO gas. An atmosphere that includes appropriate levels of CO gas can be created, for example, by providing a vessel containing a pressurized gas comprising CO gas, and releasing the pressurized gas from the vessel into a chamber or space to form an atmosphere that includes the CO gas inside the chamber or space. Alternatively, the gases can be released into an apparatus that culminates in a breathing mask or breathing tube, thereby creating an atmosphere comprising CO gas in the breathing mask or breathing tube, ensuring the patient is the only person in the room exposed to significant levels of CO.

CO levels in an atmosphere can be measured or monitored using any method known in the art. Such methods include electrochemical detection, gas chromatography, radioisotope counting, infrared absorption, colorimetry, and electrochemical methods based on selective membranes (see, e.g., Sunderman *et al.*, Clin. Chem. 28:2026-2032, 1982; Ingi *et al.*, Neuron 16:835-842, 1996). Sub-parts per million CO levels can be detected by, e.g., gas chromatography and radioisotope counting. Further, it is known in the art that CO levels in the sub-ppm range can be measured in biological tissue by a midinfrared gas sensor (see, e.g., Morimoto *et al.*, Am. J. Physiol. Heart. Circ. Physiol 280:H482-H488, 2001). CO sensors and gas detection devices are widely available from many commercial sources.

Preparation of Liquid Compositions

A CO composition may also be a liquid CO composition. A liquid can be made into a CO composition by any method known in the art for causing gases to become dissolved in liquids. For example, the liquid can be placed in a so-called "CO_2 incubator" and exposed to a continuous flow of CO, preferably balanced with carbon dioxide, until a desired concentration of CO is reached in the liquid. As another example, CO gas can be "bubbled" directly into the liquid until the desired concentration of CO in the liquid is reached. The amount of CO that can be dissolved in a given aqueous solution increases with decreasing temperature. As still another example, an appropriate liquid may be passed through tubing that allows gas diffusion, where the tubing runs through an atmosphere comprising CO (e.g., utilizing a device such as an extracorporeal membrane oxygenator). The CO diffuses into the liquid to create a liquid CO composition.

It is likely that such a liquid composition intended to be introduced into a living animal will be at or about 37°C at the time it is introduced into the animal.

The liquid can be any liquid known to those of skill in the art to be suitable for administration to patients (see, for example, Oxford Textbook of Surgery, Morris and Malt, Eds., Oxford University Press (1994)). In general, the liquid will be an aqueous solution. Examples of solutions include Phosphate Buffered Saline (PBS), Celsior™, Perfadex™, Collins solution, citrate solution, and University of Wisconsin (UW) solution (Oxford Textbook of Surgery, Morris and Malt, Eds., Oxford University Press (1994)). In one embodiment of the present invention, the liquid is Ringer's Solution, e.g., lactated Ringer's Solution, or any other liquid that can be used infused into a patient. In another embodiment, the liquid includes blood, e.g., whole blood.

Any suitable liquid can be saturated to a set concentration of CO via gas diffusers. Alternatively, pre-made solutions that have been quality controlled to contain set levels of CO can be used. Accurate control of dose can be achieved via measurements with a gas permeable, liquid impermeable membrane connected to a CO analyzer. Solutions can be saturated to desired effective concentrations and maintained at these levels.

Treatment of Patients with Carbon Monoxide Compositions

A patient can be treated with a CO composition by any method known in the art of administering gases and/or liquids to patients. CO compositions can be administered to a patient diagnosed with, or determined to be at risk for, NEC, e.g., newborns or premature infants. The present invention contemplates the systemic administration of liquid or gaseous CO compositions to patients (e.g., by inhalation and/or ingestion), and the topical administration of the compositions to the patient's gastrointestinal tract (e.g., by ingestion, insufflation, and/or introduction into the abdominal cavity).

Systemic Delivery of Carbon Monoxide

Gaseous CO compositions can be delivered systemically to a patient, e.g., a patient diagnosed with, or determined to be at risk for NEC. Gaseous CO compositions are typically administered by inhalation through the mouth or nasal passages to the lungs, where the CO is readily absorbed into the patient's bloodstream. The concentration of active compound (CO) utilized in the therapeutic gaseous composition will depend on absorption, distribution, inactivation, and excretion (generally, through respiration) rates of the CO as well as other factors known to those of skill in the art. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Acute, sub-acute and chronic administration of CO are contemplated by the present invention, depending upon, *e.g.*, the severity or persistence of NEC in the patient. CO can be delivered to the patient for a time (including indefinitely) sufficient to treat the condition and exert the intended pharmacological or biological effect.

The following are examples of some methods and devices that can be utilized to administer gaseous CO compositions to patients.

Ventilators

Medical grade CO (concentrations can vary) can be purchased mixed with air or another oxygen-containing gas in a standard tank of compressed gas (e.g., 21% O_2, 79% N_2). It is non-reactive, and the concentrations that are required for the methods of

the present invention are well below the combustible range (10% in air). In a hospital setting, the gas presumably will be delivered to the bedside where it will be mixed with oxygen or house air in a blender to a desired concentration in ppm (parts per million). The patient will inhale the gas mixture through a ventilator, which will be set to a flow rate based on patient comfort and needs. This is determined by pulmonary graphics (i.e., respiratory rate, tidal volumes etc.). Fail-safe mechanism(s) to prevent the patient from unnecessarily receiving greater than desired amounts of CO can be designed into the delivery system. The patient's CO level can be monitored by studying (1) carboxyhemoglobin (COHb), which can be measured in venous blood, and (2) exhaled CO collected from a side port of the ventilator. CO exposure can be adjusted based upon the patient's health status and on the basis of the markers. If necessary, CO can be washed out of the patient by switching to 100% O_2 inhalation. CO is not metabolized; thus, whatever is inhaled will ultimately be exhaled except for a very small percentage that is converted to CO_2. CO can also be mixed with any level of O_2 to provide therapeutic delivery of CO without consequential hypoxic conditions.

Face Mask and Tent

A CO-containing gas mixture is prepared as above to allow passive inhalation by the patient using a facemask or tent. The concentration inhaled can be changed and can be washed out by simply switching over to 100% O_2. Monitoring of CO levels would occur at or near the mask or tent with a fail-safe mechanism that would prevent too high of a concentration of CO from being inhaled.

Portable inhaler

Compressed CO can be packaged into a portable inhaler device and inhaled in a metered dose, for example, to permit intermittent treatment of a recipient who is not in a hospital setting. Different concentrations of CO could be packaged in the containers. The device could be as simple as a small tank (e.g., under 5 kg) of appropriately diluted CO with an on-off valve and a tube from which the patient takes a whiff of CO according to a standard regimen or as needed.

Intravenous Artificial Lung

An artificial lung (a catheter device for gas exchange in the blood) designed for O_2 delivery and CO_2 removal can be used for CO delivery. The catheter, when implanted, resides in one of the large veins and would be able to deliver CO at given concentrations either for systemic delivery or at a local site. The delivery can be a local delivery of a high concentration of CO for a short period of time at the site of the procedure, e.g., in proximity to the small intestine (this high concentration would rapidly be diluted out in the bloodstream), or a relatively longer exposure to a lower concentration of CO (see, e.g., Hattler *et al.,* Artif. Organs 18(11):806-812 (1994); and Golob *et al.,* ASAIO J., 47(5):432-437 (2001)).

Normobaric chamber

In certain instances, it would be desirable to expose the whole patient to CO. The patient would be inside an airtight chamber that would be flooded with CO (at a level that does not endanger the patient, or at a level that poses an acceptable risk without the risk of bystanders being exposed. Upon completion of the exposure, the chamber could be flushed with air (e.g., 21% O_2, 79% N_2) and samples could be analyzed by CO analyzers to ensure no CO remains before allowing the patient to exit the exposure system.

Systemic Delivery of Liquid CO Compositions

The present invention further contemplates that liquid CO compositions can be created for systemic delivery to a patient, e.g., by infusion into a patient. For example, liquid CO compositions, such as CO-saturated Ringer's Solution, can be infused into a patient suffering from or at risk for NEC. Alternatively or in addition, CO-partially or completely saturated whole (or partial) blood can be infused into the patient. The present invention also contemplates that agents capable of delivering doses of CO gas or liquid can be utilized (e.g., CO releasing gums, creams, ointments or patches).

Topical Treatment of the Gastrointestinal Tract with Carbon Monoxide

Alternatively or in addition, CO compositions can be applied directly to the gastrointestinal tract, e.g., to the interior and/or exterior of the entire gastrointestinal tract, or to any portion thereof. A gaseous composition can be directly applied to the

gastrointestinal tract of a patient, e.g., a premature infant or newborn, by any method known in the art for insufflating gases into a patient. For example, gases, e.g., carbon dioxide, are often insufflated into the gastrointestinal tract and the abdominal cavity of patients to facilitate examination during endoscopic and laproscopic procedures, respectively (see, e.g., Oxford Textbook of Surgery, Morris and Malt, Eds., Oxford University Press (1994)). The skilled practitioner will appreciate that similar procedures could be used to administer CO compositions directly to the gastrointestinal tract of a patient. It is also contemplated that the present invention can be applied to help prevent NEC resulting from laproscopy and endoscopy, e.g., colonoscopy and oesophagogastroduodenoscopy.

Aqueous CO compositions can also be administered topically to the gastrointestinal tract of a patient. Aqueous forms of the compositions can be administered by any method known in the art for administering liquids to patients. As with gaseous compositions, aqueous compositions can be applied directly to the interior and/or exterior of the gastrointestinal tract. For example, the aqueous form can be administered orally, e.g., by causing the patient to ingest an encapsulated or unencapsulated dose of the aqueous CO composition. As another example, liquids, e.g., saline solutions containing dissolved CO, can be injected into the gastrointestinal tract and the abdominal cavity of patients during endoscopic and laproscopic procedures, respectively. Further, an *in situ* exposure can be carried out by flushing the gastrointestinal tract or a portion thereof with a liquid CO composition (see Oxford Textbook of Surgery, Morris and Malt, Eds., Oxford University Press (1994)).

Use of Hemoxygenase-1, Other Compounds, and Other Treatments for NEC

Also contemplated by the present invention is the induction or expression of hemeoxygenase-1 (HO-1) in conjunction with administration of CO. For example, HO-1 can be induced in a patient suffering from or at risk for NEC. As used herein, the term "induce(d)" means to cause increased production of a protein, e.g., HO-1, in isolated cells or the cells of a tissue, organ or animal using the cells' own endogenous (e.g., non-recombinant) gene that encodes the protein.

HO-1 can be induced in a patient by any method known in the art. For example, production of HO-1 can be induced by hemin, by iron protoporphyrin, or by cobalt protoporphyrin. A variety of non-heme agents including heavy metals, cytokines,

hormones, NO, $COCl_2$, endotoxin and heat shock are also strong inducers of HO-1 expression (Choi *et al.,* Am. J. Respir. Cell Mol. Biol. 15:9-19, 1996; Maines, Annu. Rev. Pharmacol. Toxicol. 37:517-554, 1997; and Tenhunen *et al.,* J. Lab. Clin. Med. 75:410-421, 1970). HO-1 is also highly induced by a variety of agents causing oxidative stress, including hydrogen peroxide, glutathione depletors, UV irradiation, endotoxin and hyperoxia (Choi *et al.,* Am. J. Respir. Cell Mol. Biol. 15:9-19, 1996; Maines, Annu. Rev. Pharmacol. Toxicol. 37:517-554, 1997; and Keyse *et al.,* Proc. Natl. Acad. Sci. USA 86:99-103, 1989). A "pharmaceutical composition comprising an inducer of HO-1" means a pharmaceutical composition containing any agent capable of inducing HO-1 in a patient, e.g., any of the agents described above, e.g., hemin, iron protoporphyrin, and/or cobalt protoporphyrin.

HO-1 expression in a cell can be increased via gene transfer. As used herein, the term "express(ed)" means to cause increased production of a protein, e.g., HO-1 or ferritin, in isolated cells or the cells of a tissue, organ or animal using an exogenously administered gene (e.g., a recombinant gene). The HO-1 or ferritin is preferably of the same species (e.g., human, mouse, rat, etc.) as the recipient, in order to minimize any immune reaction. Expression could be driven by a constitutive promoter (e.g., cytomegalovirus promoters) or a tissue-specific promoter (e.g., milk whey promoter for mammary cells or albumin promoter for liver cells). An appropriate gene therapy vector (e.g., retrovirus, adenovirus, adeno associated virus (AAV), pox (e.g., vaccinia) virus, human immunodeficiency virus (HIV), the minute virus of mice, hepatitis B virus, influenza virus, Herpes Simplex Virus-1, and lentivirus) encoding HO-1 or ferritin would be administered to a patient suffering from or at risk for NEC, by mouth, by inhalation, or by injection into the intestinal wall, intestinal lumen, or abdominal cavity. Similarly, plasmid vectors encoding HO-1 or apoferritin can be administered, e.g., as naked DNA, in liposomes, or in microparticles.

Further, exogenous HO-1 protein can be directly administered to a patient by any method known in the art. Exogenous HO-1 can be directly administered in addition, or as an alternative, to the induction or expression of HO-1 in the patient as described above. The HO-1 protein can be delivered to a patient, for example, in liposomes, and/or as a fusion protein, e.g., as a TAT-fusion protein (see, e.g., Becker-Hapak *et al.*, Methods 24:247–256, 2001).

Alternatively or in addition, any of the products of metabolism by HO-1, e.g., bilirubin, biliverdin, iron, and/or ferritin, can be administered to a patient in conjunction with CO in order to prevent or treat NEC. Further, the present invention contemplates that iron-binding molecules other than ferritin, e.g., desferoxamine (DFO), iron dextran, and/or apoferritin, can be administered to the patient. Further still, the present invention contemplates that enzymes (e.g., biliverdin reductase) that catalyze the breakdown any of these products can be inhibited to create/enhance the desired effect. Any of the above can be administered, e.g., orally, intravenously, intraperitoneally, or by direct administration to the inside or outside of the bowel.

The present invention contemplates that compounds that release CO into the body after administration of the compound (e.g., CO-releasing compounds, e.g., photoactivatable CO-releasing compounds), e.g., dimanganese decacarbonyl, tricarbonyldichlororuthenium (II) dimer, and methylene chloride (e.g., at a dose of between 400 to 600 mg/kg, e.g., about 500mg/kg), can also be used in the methods of the present invention, as can carboxyhemoglobin and CO-donating hemoglobin substitutes.

The above can be administered to a patient in any way, e.g., by oral, intraperitoneal, intravenous, or intraarterial administration. Any of the above compounds can be administered to the patient locally and/or systemically, and in any combination.

The present invention further contemplates treating NEC by administering CO to the patient in combination with any other known methods or compounds for treating NEC, e.g., cessation or reduction of the rate of feeding by mouth, e.g., for at least 1 day, e.g., at least 2, 3, 5, or 10 days, or more); administering intravenous hydration and/or nutrition, nasogastric decompression, or antimicrobial agents to the patient; surgical intervention; and/or draining the patient's peritoneal cavity. Surgical interventions include, e.g., resecting the affected portion of the bowel. Also contemplated is preventive treatment of patients at risk for NEC by administering CO to the patient in combination with any other known methods for preventing NEC, e.g., changing the patient's diet.

The invention is illustrated in part by the following examples, which are not to be taken as limiting the invention in any way.

Example 1. CO attenuates the development of NEC

Collection of human intestinal specimens

Human intestinal specimens were collected and snap frozen at the time of operation.

Animal model of necrotizing enterocolitis

Pregnant time-dated Sprague-Dawley rats were induced at term using subcutaneous injection of Pitocin (1 U). Immediately after birth (day zero), the neonates were weighed and randomized into two main groups. Animals were either left with their mothers and thus breast-fed, or separated from their mothers, housed in an incubator (Ohio Medical Products, Madison, WI), gavage fed with a special rodent formula two times daily, and subjected to 10 minutes of hypoxia (5% O_2, 95% N_2; PraxAir, Pittsburgh, PA) prior to each feeding. The formula consisted of 15 g Similac® 60/40 (Ross Pediatrics, Colombus, OH) in 75 mL of Esbilac® canine milk replacement (Pet-Ag Inc., Hampshire, IL). Rats from each group were further randomized to receive no additional treatment or one-hour per day CO treatment (250 parts per million; ppm, days 1-3). CO was delivered as described below. The neonatal rats were sacrificed on day four. The intestines were inspected for gross necrotic changes and pneumatosis intestinalis. The last 2 cm of terminal ileum was harvested for morphological studies, and mucosal scrapings were collected for detection of protein.

CO exposure

Animals were exposed to CO at a concentration of 250 ppm. Briefly, 1% CO in air was mixed with air (21% oxygen) in a stainless steel mixing cylinder and then directed into a 3.70 ft^3 glass exposure chamber at a flow rate of 12 L/min. A CO analyzer (Interscan, Chatsworth, CA) was used to measure CO levels continuously in the chamber. CO concentrations were maintained at 250 ppm at all times. Rats were placed in the exposure chamber as required.

Morphological studies

Intestinal specimens were harvested as described above. Hematoxylin and eosin (H&E) slides were prepared as per standard protocol and examined by light microscopy. The presence of morphological changes in the intestinal epithelium, including separation of the villous core, submucosal edema, and epithelial sloughing, were determined by a pathologist in a blinded fashion.

Serum cytokine determination

Serum was collected for determination of rat TNF-α and IL-1β levels using Quantikine® ELISA (R&D systems, Minneapolis, MN) per the manufacturer's instructions.

Cell culture

The rat small-intestinal epithelial cell line IEC-6 was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (Bio-Whittaker, Walkersville, MD) supplemented with 5% fetal bovine serum, 0.02 mM glutamine (Gibco, Grand Island, NY), 0.1 U/mL insulin, and 100 U/mL penicillin/100 μg/mL streptomycin at 37°C and 10% CO_2. Cells from passages 3 through 20 were used for experiments.

Viability

Cell viability was determined by measuring ATP levels (CellTiter-Glo™; Promega) as per the manufacturer's protocol.

Western blot analysis

IEC-6 cells or ileal mucosal scrapings were collected in lysis buffer containing 20 mmol/L Tris with 100 μmol/L phenylmethylsulfonylfluoride (Sigma), 1 μmol/L leupeptin (Sigma), and 1 μmol/L sodium orthovanadate (Sigma). Protein was quantified by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Lysates (30 μg) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Reporter assay

pGL3/2 rat iNOS promoter-luciferase reporter was constructed as described in Beck et al., FEBS Lett 435:35-38 (1998). IEC-6 cells grown in 35mm wells were transfected with 4μL Lipofectamine2000® (Invitrogen), 0.15μg pGL3/2 DNA and 0.5μg pIEP-LacZ DNA, which was used as a control for transfection efficiency. Twenty-four hours following transfection, cells were treated for 6 hours, then lysates were collected and a luciferase assay (Promega) was performed with a luminometer (Berthold, Germany).

Nitrite determination

Nitrite (NO_2^-) was measured in the culture medium using the Griess method.

Statistical analysis

Results are expressed as mean ± SEM. Differences among groups were analyzed with one-way analysis of variance with Student-Newman-Keuls post-hoc test for all pairwise comparisons (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when P was less than 0.05.

Intestinal HO-1 protein is increased in NEC

Human intestinal specimens from patients with NEC and control intestinal specimens from patients with non-inflammatory conditions were analyzed for expression of HO-1. Whole cell lysates from NEC specimens demonstrated increased expression of HO-1 compared to controls (Fig. 1A).

To determine whether intestinal HO-1 protein levels would be increased in a model of experimental NEC, neonatal rats were randomized to breast feed ad libitum or to be subjected to intermittent hypoxia and formula fed (H/F) as described above. All animals were sacrificed on day 4 of life and terminal ileal mucosa were collected and analyzed by Western blotting. HO-1 protein expression was increased in the H/F group compared to breast fed control animals (Fig. 1B). This is consistent with previous findings in the intestine and other organs that illustrate upregulation of HO-1 following a variety of injuries.

CO protects against the development of NEC

Neonatal rats from breast fed and H/F groups were randomized to receive either one-hour doses of CO (250 ppm) per day on days 1, 2, and 3, or no additional therapy. All animals were sacrificed on day 4 of life. Gross pathological characteristics including *pneumatosis intestinalis* and necrosis (see Table 1, below) as well as histological changes consistent with experimental NEC were identified in the H/F group compared to breast fed controls (Fig. 2A, 2B, 2C, and 2D). CO treatment had no effect on gross or microscopic evaluation of breast fed animals and significantly protected against the development of experimental NEC in rats in the H/F group. TUNEL staining for cell death, which was increased in the H/F group, was diminished by CO treatment (Fig. 3A, 3B, 3C, and 3D).

Table 1. Pathological changes from neonatal rat ileum on day 4 of life.

	Breast-fed	Breast-fed + CO	Formula-Fed/Hypoxia	Formula-Fed/ Hypoxia + CO
Total number (*n*)	7	7	8	8
Gross Changes (*n*)				
Bowel wall necrosis	0	0	5	2
Pneumatosis intestinalis	0	0	5	1
Microscopic Changes (*n*)				
Villous atrophy	0	0	7	2
Vacuolization	0	1	6	3
Stromal neutrophils	0	0	6	1

CO decreases systemic markers of inflammation.

At the time of sacrifice, serum was collected for measurement of TNF-α and IL-1β levels as systemic markers of tissue inflammation. Levels of both TNF-α and IL-1β were increased by 33.2 and 18.5 fold in the H/F group compared to breast fed controls (Figs. 4A and 4B; $P<0.05$). CO significantly attenuated these increases, resulting in only 3 and 2.5 fold increases in TNF-α and IL-1β, respectively ($P<0.05$). These findings demonstrate that exogenous CO can abrogate some of the systemic consequences in this animal model.

CO decreases local intestinal markers of inflammation.

Assays were performed for cyclooxygenase-2 (COX-2) and interleukin-1β (IL-1β), both of which are markers of intestinal inflammation and have been demonstrated to be associated with NEC. Ileal mucosal protein lysates were evaluated by Western blot analysis for COX-2 and IL-1β. In this study H/F was associated with an increase in ileal COX-2 and IL-1β protein levels in approximately half of the animals (Fig. 5). Consistent with the protective effects on histology and serum cytokines, CO-treatment decreased expression of both of these proteins. CO also decreased expression of ileal HO-1 in this model (data not shown), which likely represents a decrease in intestinal injury and the compensatory changes that occur in response to the injury.

CO inhibits generation of intestinal inducible nitric oxide synthase (iNOS)

iNOS is increased in human intestinal specimens from neonates with NEC as well as in the intestines of rats in experimental models of NEC. Induction of iNOS and NO generation are believed to contribute directly to tissue damage via formation of reactive nitrogen species. Western blot analysis of ileal mucosal samples in this study demonstrate that H/F results in increased levels of iNOS and protein nitration (Fig. 6). Nitration or nitrotyrosine formation is thought to be caused by peroxynitrite, the toxic product of the interaction of NO and superoxide. Both iNOS protein and nitrosotyrosines were markedly decreased in animals treated with CO, suggesting that CO may be protective in the intestines by decreasing/preventing the generation of NO.

HO-1/CO inhibits IEC-6 cell death

To determine whether HO-1 or CO inhibits cell death *in vitro,* the rat intestinal epithelial cell line IEC-6 was utilized. Cell death was induced by treating these cells with TNF-α (10 ng/ml) plus actinomycin D (ActD; 200 ng/ml). Cell viability was analyzed by crystal violet staining of adherent cells and assessment of cellular ATP content. TNF-α/ActD decreased IEC-6 cell viability to 24±2.1% that of untreated controls (Fig. 7; $P<0.05$). CO significantly inhibited the TNF-α/ActD-induced cell death resulting in 54 ± 5.6% viability compared to untreated controls ($P<0.05$). CO alone had no measurable effect on viability of IEC-6 cells *in vitro*. Induction of HO-1 by CoPP had similar protective effects.

CO prevents iNOS upregulation/NO generation

Whether CO can inhibit the upregulation of iNOS intestinal epithelial cells *in vitro* was investigated. The effects of LPS and/or 1% oxygen (hypoxia) were investigated on iNOS protein by Western blotting. These studies demonstrate that LPS/hypoxia increased iNOS protein, which is an effect that was inhibited by CO (Fig. 8A). The effects of CO on transcriptional activation of the rat iNOS promoter was tested in IEC-6 cells stimulated with LPS and hypoxia (Fig. 8B). The combination of LPS plus hypoxia resulted in a 4.9 ± 0.3 fold increase in transcriptional activation of the iNOS promoter utilizing a luciferase assay ($P<0.05$). CO limited this transcriptional activation to only a 1.7 ± 0.2 fold increase($P<0.05$). Additionally, the

effect of CO on NO generation by IEC-6 cells was assayed by measuring nitrite. IEC-6 cells were stimulated with a cytokine mixture (TNF-α, IL-1β, Interferon-γ) that is known to upregulate iNOS. Cytokine stimulation increased iNOS protein (Fig. 8C) as well as nitrite to 17.2±0.9 μM compared to 1.4±0.3 μM in unstimulated controls ($P<0.01$; Fig. 8D). CO and CoPP significantly inhibited this cytokine effect resulting in nitrite levels of 9.8 ± 0.7 and 10.4 ± 1.0, respectively ($P<0.05$).

The data presented above indicate that HO-1 is upregulated in intestinal specimens from human neonates with NEC as well as in the ileal mucosa of neonatal rats in a model of experimental NEC. One-hour per day of exogenous CO delivery prevented the development of NEC. This was associated with a decrease in systemic inflammation as assayed by serum TNF-α and IL-1β, and local inflammation as assayed by ileal mucosal expression of IL-1β and COX-2. The development of NEC was also associated with increased ileal mucosal iNOS expression and protein nitration. CO therapy attenuated the increase in iNOS and nitrosative stress. *In vitro*, induction of HO-1 or CO is protective against TNF-α/ActD-induced cell death in IEC-6 cells. Additionally *in vitro*, CO prevented iNOS induction and NO generation.

HO-1 and its catalytic by-products, including CO, likely play a defensive role following intestinal injury. The data presented above demonstrate that levels of HO-1 protein are increased in the ileal mucosa of animals with NEC, which is consistent with previous studies demonstrating increased intestinal HO-1 in models of ischemia/reperfusion and hemorrhagic shock. Induction of HO-1, by pre-conditioning or pharmacologically, is cytoprotective following intestinal transplantation and ischemia/reperfusion. CO treatment, which is protective against the development of NEC, also prevented the upregulation of HO-1. This inhibition of HO-1 expression may be due to a direct negative feedback loop; however, this is likely secondary to the protection against intestinal injury with subsequent upregulation of HO-1.

One hour of CO inhalation therapy (250 ppm) per day was sufficient to prevent the development of NEC. The protective effects of CO in this animal model of NEC are likely to be multifactorial. The data presented above demonstrate that CO administration causes a decrease in intestinal apoptosis as assayed by TUNEL staining *in vivo* and amelioration of TNF-α/ActD-induced IEC-6 cell death *in vitro*. CO therapy

was also associated with decreased elaboration of both systemic and local inflammatory mediators.

iNOS/NO are associated with mucosal damage and gut barrier failure in inflammatory bowel disease, experimental ileitis, endotoxic shock and NEC. The data presented above show that CO decreases the upregulation of iNOS *in vitro* and *in vivo*, in addition to decreasing nitrosotyrosine formation *in vivo*. Peroxynitrite, which is believed to be the reactive nitrogen species responsible for enterocyte toxicity and apoptosis, reacts with proteins to result in nitration of tyrosine residues. This species is thought to be formed by the interaction of NO and superoxide. CO-mediated enterocyte protection may be mediated by inhibition of iNOS protein upregulation, decreasing subsequent NO and peroxynitrite generation.

Prevention of the development of NEC by CO may involve maintained gastrointestinal motility and prevention of inappropriate bacterial colonization. Altered intestinal colonization is known to contribute to the development of NEC.

In conclusion, the present example demonstrates that one-hour of CO therapy per day protected formula-fed/hypoxic neonatal rats from the development of experimental NEC. The mechanism appears to involve the inhibition of iNOS expression and protein nitration.

Example 2. Protocol for the Treatment of NEC

The following example illustrates protocols for use in treating a patient suffering from or at risk for NEC. The example also illustrates protocols for treating patients before, during, and/or after surgical procedures, e.g., a surgical procedure to treat NEC, e.g., a surgery in which a portion of the bowel is resected. Skilled practitioners will appreciate that any protocol described herein can be adapted based on a patient's individual needs, and can be adapted to be used in conjunction with any other treatment for NEC.

Treatment of a patient with CO can begin on the day the patient is diagnosed as suffering from NEC or any condition associated with NEC, or as having any risk factor associated with an increased likelihood that the patient will develop NEC. Patients can inhale CO at concentrations ranging from 10 ppm to 1000 ppm, e.g., about 100 ppm to about 800 ppm, about 150 ppm to about 600 ppm, or about 200 ppm to about 500 ppm. Preferred concentrations include, e.g., about 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125

ppm, 200 ppm, 250 ppm, 500 ppm, 750 ppm, or about 1000 ppm. CO can be administered to the patient intermittently or continuously. CO can be administered for about 1, 2, 4, 6, 8, 10, 12, 14, 18, or 20 days, or greater than 20 days, e.g., 1 2, 3, 5, or 6 months, or until the patient no longer exhibits symptoms of NEC, or until the patient is diagnosed as no longer being at risk for NEC. In a given day, CO can be administered continuously for the entire day, or intermittently, e.g., a single whiff of CO per day (where a high concentration is used), or for up to 23 hours per day, e.g., up to 20, 15, 12, 10, 6, 3, or 2 hours per day, or up to 1 hour per day.

With regard to the administration of CO in conjunction with surgical procedures to treat NEC, CO can be administered systemically or locally to a patient prior to, during, and/or after a surgical procedure is performed. Patients can inhale CO at concentrations ranging from 10 ppm to 1000 ppm, e.g., about 100 ppm to about 800 ppm, about 150 ppm to about 600 ppm, or about 200 ppm to about 500 ppm. Preferred concentrations include, e.g., about 30 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 200 ppm, 250 ppm, 500 ppm, 750 ppm, or about 1000 ppm. CO can be administered to the patient intermittently or continuously, for about 1, 2, 4, 6, 8, 10, 12, 14, 18, or 20 days, or greater than 20 days, before the procedure. Alternatively or in addition, CO can be administered to the patient during the procedure, e.g., by inhalation and/or topical administration. Alternatively or in addition, CO can be administered to the patient after the procedure, e.g., starting immediately after completion of the procedure, and continuing for about 1, 2, 3, 5, 7, or 10 hours, or about 1, 2, 5, 8, 10, 20, 30, 50, or 60 days, indefinitely, or until the patient no longer suffers from, or is at risk for, NEC after the completion of the procedure.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating necrotizing enterocolitis in a patient, comprising:

identifying a patient suffering from or at risk for necrotizing enterocolitis; and

administering to the patient a pharmaceutical composition comprising an amount of carbon monoxide effective to treat necrotizing enterocolitis in the patient.

2. The method of claim 1, wherein the pharmaceutical composition is administered to the patient via inhalation.

3. The method of claim 1, wherein the pharmaceutical composition is in liquid form and is administered to the patient orally.

4. The method of claim 1, wherein the pharmaceutical composition is administered directly to the abdominal cavity of the patient.

5. The method of claim 1, wherein the patient is an infant.

6. The method of claim 5, wherein the patient is a premature infant.

7. The method of claim 1, wherein the patient exhibits low birth weight.

8. The method of claim 1, wherein the patient exhibits hypoxia.

9. The method of claim 1, wherein the patient exhibits hypothermia.

10. The method of claim 1, wherein the patient exhibits hypotension.

11. The method of claim 1, wherein the patient exhibits hyperviscosity of the blood.

12. The method of claim 1, wherein the patient exhibits acidosis.

13. The method of claim 1, wherein the patient has received an exchange transfusion.

14. The method of claim 1, wherein the patient has received at least one hyperosmolar feed.

15. The method of claim 1, wherein the patient has received a packed cell transfusion.

16. The method of claim 1, wherein the patient has received an overdosage of calcium antagonists.

17. The method of claim 1, wherein the patient suffers from mesenteric ischaemia.

18. The method of claim 1, wherein the patient suffers from a bacterial infection of the bowel wall.

19. The method of claim 1, wherein the patient has undergone surgery.

20. The method of claim 1, wherein the patient is about to undergo surgery.

21. The method of claim 1, wherein the patient is undergoing surgery.

22. The method of claim 1, wherein the pharmaceutical composition is in gaseous form.

23. The method of claim 1, further comprising administering to the patient a treatment selected from the group consisting of: intravenous nutrition; intravenous hydration; antimicrobial agents; performing nasogastric decompression on the patient, performing surgery on the patient; and draining the patient's peritoneal cavity.

24. A method of treating necrotizing enterocolitis in a patient, comprising:

(a) identifying a patient suffering from necrotizing enterocolitis;

(b) performing surgery on the patient to resect an affected portion of the patient's bowel; and

(c) administering to the patient a pharmaceutical composition comprising an amount of carbon monoxide effective to treat necrotizing enterocolitis in the patient after (a) and before, during, or after (b).

25. The method of claim 1, wherein the pharmaceutical composition is administered by artificial lung.

26. The method of claim 1, wherein the pharmaceutical composition is administered by an extracorporeal membrane gas exchange device.

27. A method of treating necrotizing enterocolitis in a patient, comprising:

(a) identifying a patient suffering from or at risk for necrotizing enterocolitis;

(b) providing a vessel containing a pressurized gas comprising carbon monoxide gas;

(c) releasing the pressurized gas from the vessel, to form an atmosphere comprising carbon monoxide gas; and

(d) exposing the patient to the atmosphere, wherein the amount of carbon monoxide in the atmosphere is sufficient to treat necrotizing enterocolitis in the patient.

28. A vessel comprising medical grade compressed carbon monoxide gas, the vessel bearing a label indicating that the gas can be used to treat necrotizing enterocolitis in a patient.

29. The vessel of claim 28, wherein the carbon monoxide gas is in admixture with an oxygen-containing gas.

30. The vessel of claim 29, wherein the carbon monoxide gas is present in the admixture at a concentration of at least about 0.025%.

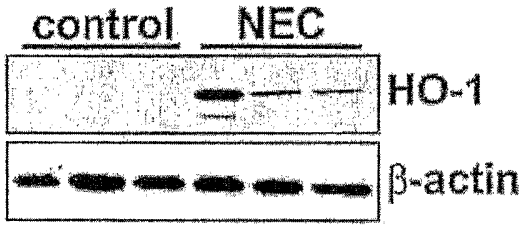
31. The vessel of claim 29, wherein the carbon monoxide gas is present in the admixture at a concentration of at least about 0.05%.

32. The vessel of claim 29, wherein the carbon monoxide gas is present in the admixture at a concentration of at least about 0.10%.

33. The vessel of claim 29, wherein the carbon monoxide gas is present in the admixture at a concentration of at least about 1.0%.

34. The vessel of claim 29, wherein the carbon monoxide gas is present in the admixture at a concentration of at least about 2.0%.

A.



B.

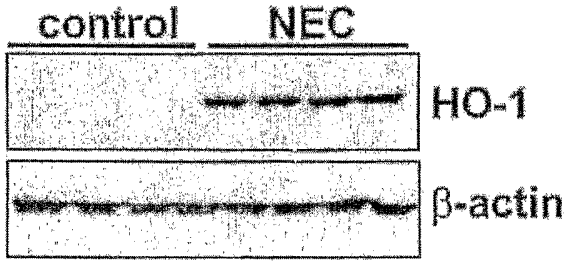


Fig. 1A-1B

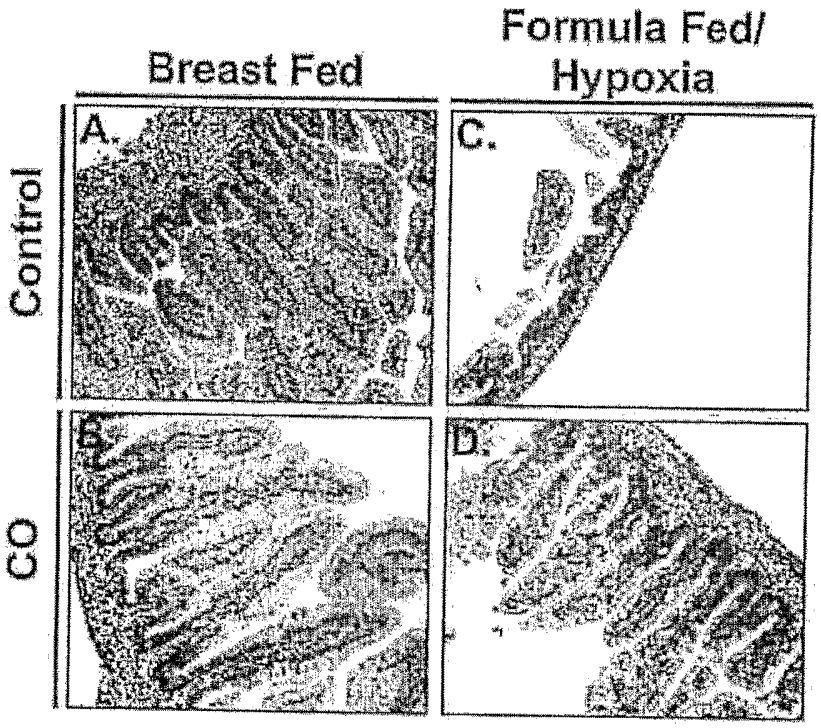


Fig. 2A-2D

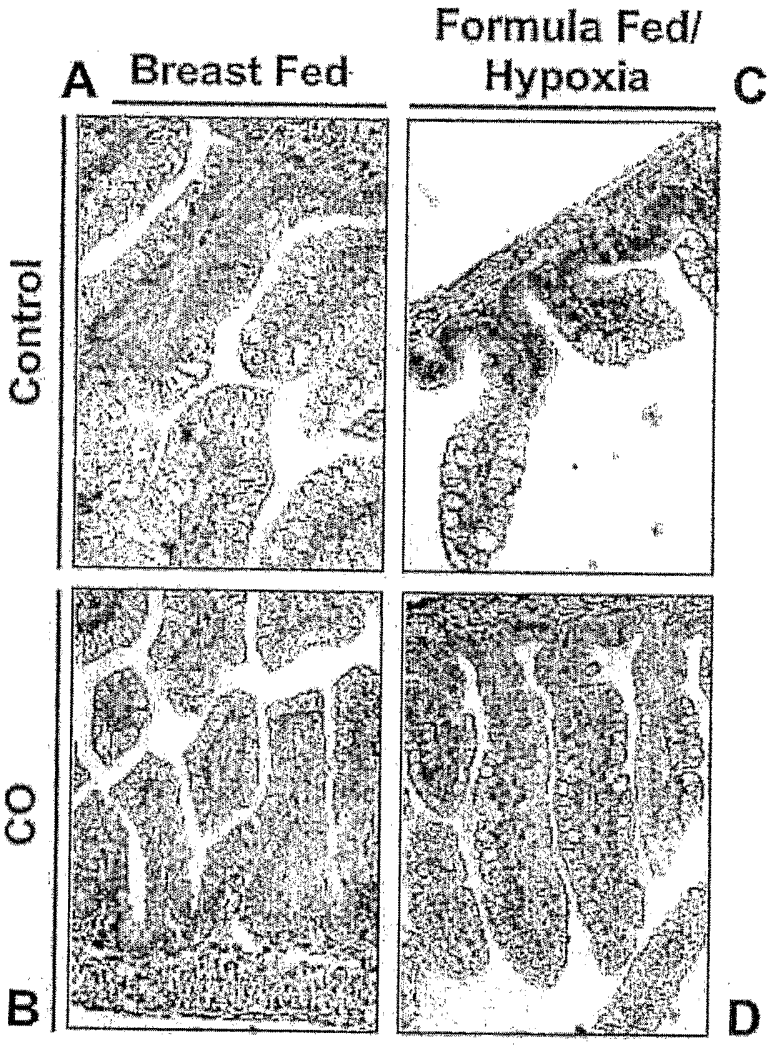
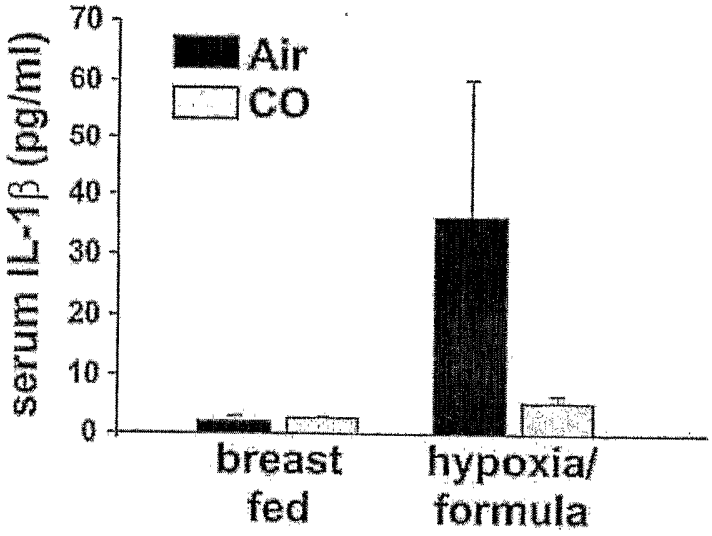


Fig. 3A-3D

A.


serum IL-1β (pg/ml)
70
60
50
40
30
20
10
0
Air
CO
breast fed
hypoxia/ formula

B.

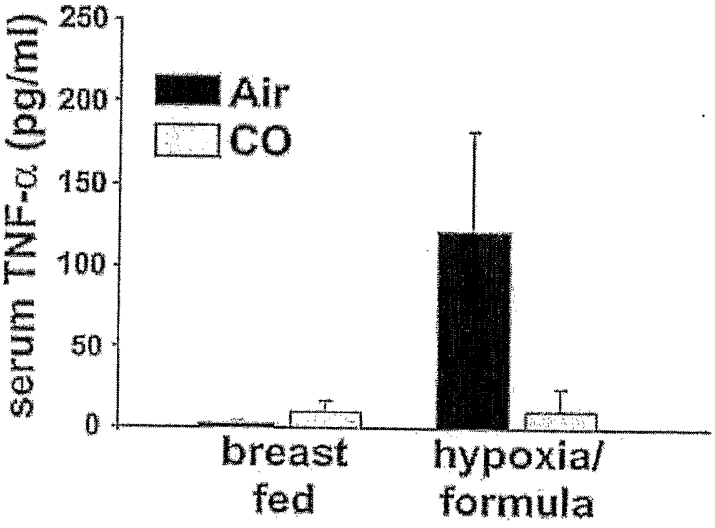

serum TNF-α (pg/ml)
250
200
150
100
50
0
Air
CO
breast fed
hypoxia/ formula

Fig. 4A-4B

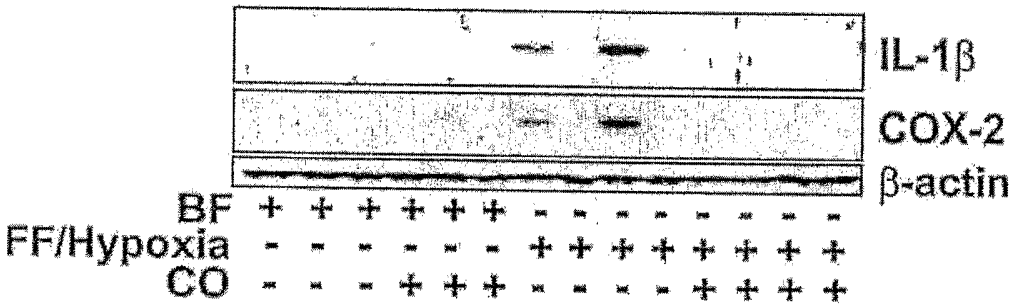


Fig. 5

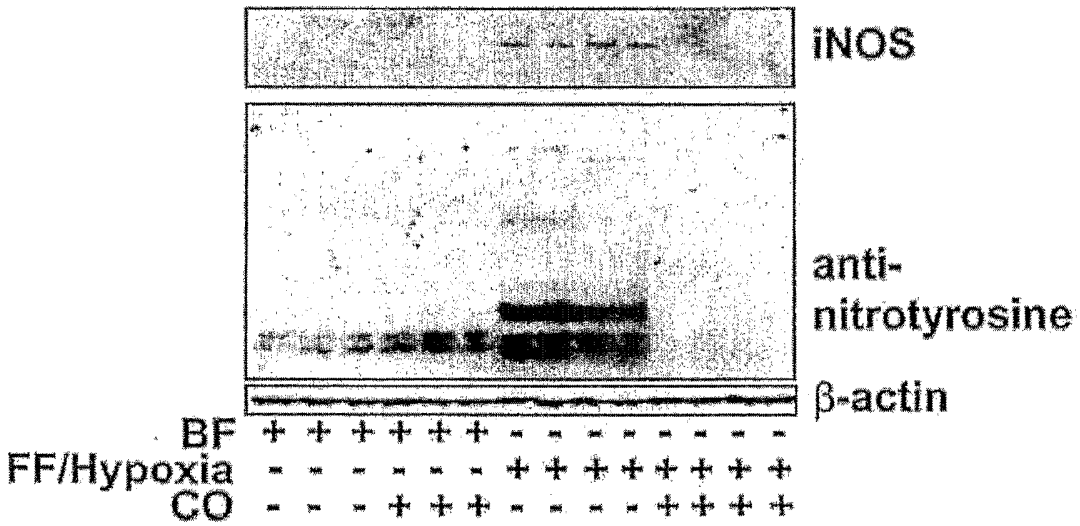


Fig. 6

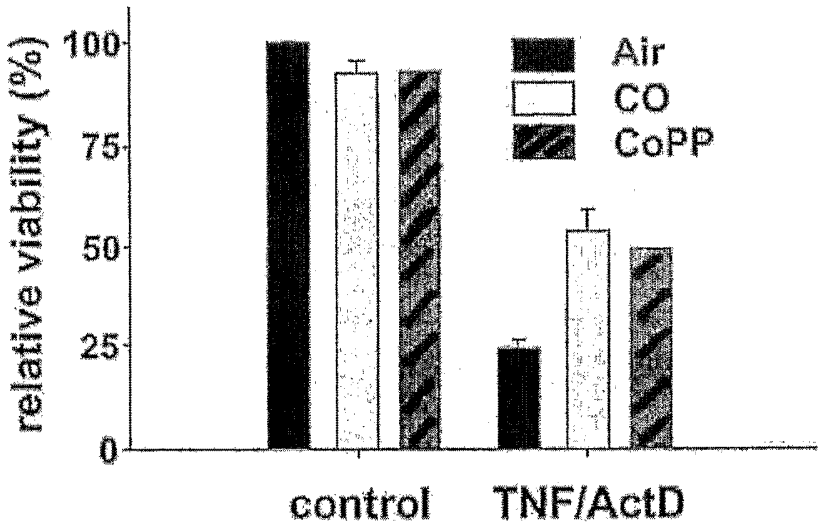
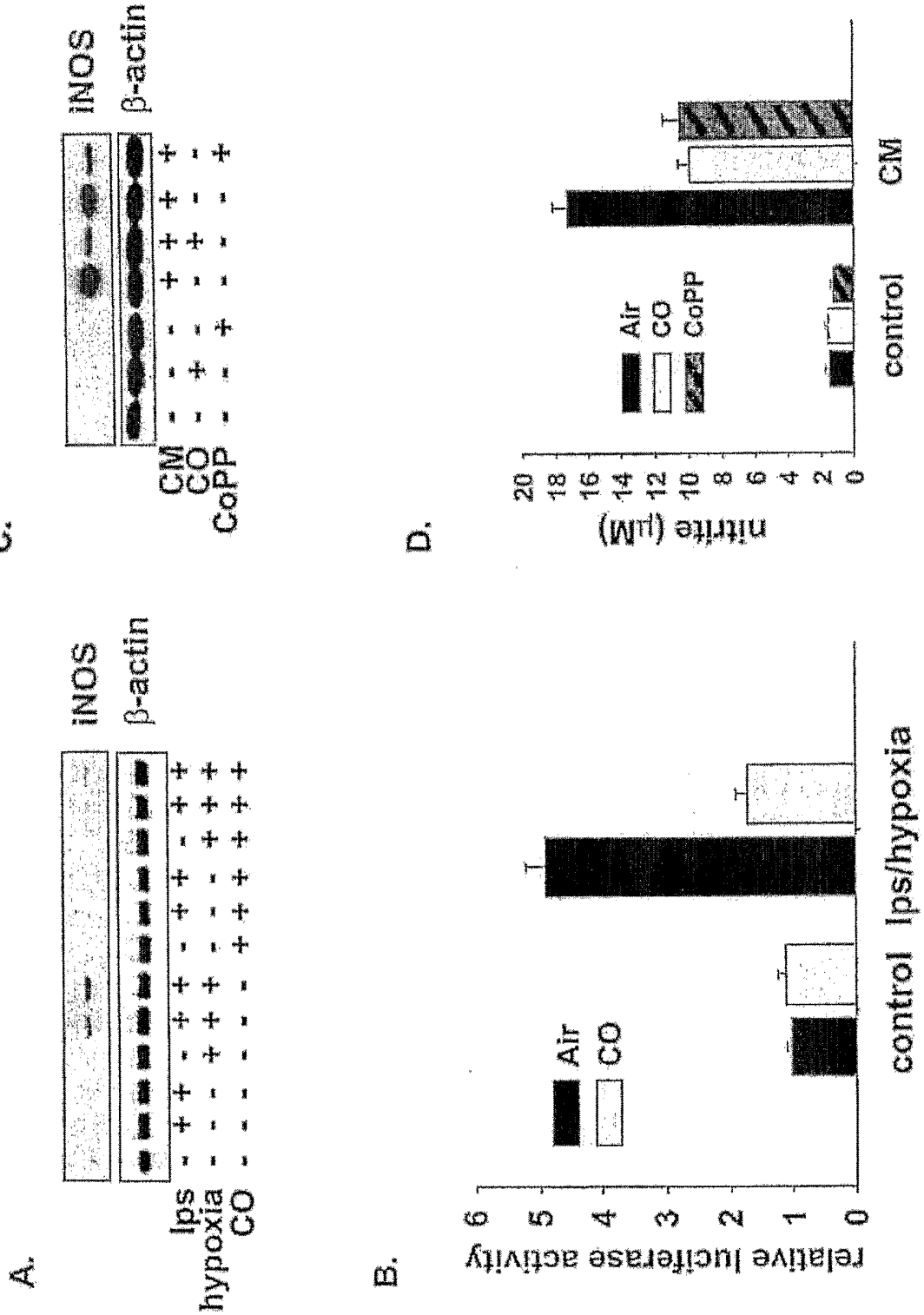

relative viability (%)
100
75
50
25
0
Air
CO
CoPP
control
TNF/ActD

Fig. 7


A.
iNOS
β-actin
lps
hypoxia
CO
B.
relative luciferase activity
Air
CO
control
lps/hypoxia
C.
iNOS
β-actin
CM
CO
CoPP
D.
nitrite (μM)
Air
CO
CoPP
control
CM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/11167

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 33/00, 47/00
US CL : 424/699; 514/771

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/699; 514/771

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,792,325 A (Richardson) 11 August 1998, entire reference.	28-34
Y	Database JPAB on WEST. ACCESSION NO. 1981-59741D, 'Yersinia entercolitica polysaccharide-sensitised blood corpuscles', JP56079957, 30 June 1981, see abstract.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"E" earlier application or patent 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)

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2003 (21.07.2003)	13 AUG 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Valerie Bell-Harris for Alton Pryor Telephone No. 703 308-1235

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