(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 161 952 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
12.12.2001 Bulletin 2001/50

(51) Int Cl.[7]: **A61K 38/17**, A61P 31/00

(21) Application number: **01121005.1**

(22) Date of filing: **24.01.1995**

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **24.01.1994 US 188221**
16.08.1994 US 291112

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
95908723.0 / 0 741 575

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Remarks:
This application was filed on 31 - 08 - 2001 as a divisional application to the application mentioned under INID code 62.

(54) Human therapeutic uses of BPI protein products

(57) Disclosed are methods for treatment of humans exposed to bacterial endotoxin in circulation by administration of bactericidal/permeability-increasing (BPI) protein products. Serologically and hematologically verifiable alleviation of endotoxin mediated increases in circulating cytokines, fibrinolysis and coagulation factors and changes in lymphocyte counts are observed upon such treatment. Also observed is alleviation of endotoxin mediated decreases in systemic vascular resistance index (SVRI) and concomitant increases in cardiac index (CI).

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Description

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a continuation-in-part of U.S. Patent Application Serial No. 08/291,112 filed August 16, 1994 which is a continuation-in-part of U.S. Patent Application Serial No. 08/188,221, filed January 24, 1994.

BACKGROUND OF THE INVENTION

[0002] The present invention relates generally to therapeutic methods and more particularly to methods for treatment of humans exposed to bacterial endotoxin in blood circulation as a result of, *e.g.*, Gram negative bacterial infection, accidental injection of endotoxin-contaminated fluids, translocation of endotoxin from the gut, release of endotoxin into circulation as a result of antibiotic mediated bacterial cytolysis and the like.

[0003] Gram negative bacterial endotoxin plays a central role in the pathogenesis of gram negative sepsis and septic shock, conditions which remain leading causes of morbidity and death in critically ill patients. Endotoxin interacts with inflammatory cells, releasing endogenous mediators such as cytokines, hydrolases, peptides, prostaglandins and other compounds that contribute to the pathophysiology of septic shock. While principal causes of entry of endotoxin into circulation are Gram negative bacteremia and translocation of bacteria and bacterial products from the gut, endotoxin also may enter circulation as the result of accidental injection of contaminated fluids or through release of endotoxin from bacteria lysed as a consequence of antibiotic therapy. In the recent past, therapeutic methods proposed for treatment of sepsis and septic shock have had as their focus attempts to bind endotoxin in circulation and inhibit direct and indirect release into circulation of proinflammatory substances mediated by the presence of endotoxin. Anti-endotoxin antibodies, for example, have shown promise in inhibition of endotoxin effects in humans.

[0004] A difficulty consistently encountered in developing therapeutic methods and materials for treatment of endotoxemia in humans has been the general unreliability of *in vitro* and even non-human *in vivo* test results as an indicator of human therapeutic potential. Because the effects of endotoxin in circulation are complex and involve direct and indirect responses by many cell types in the body, test results in attempts to intervene in endotoxin's effects on a particular cell type *in vitro* present an incomplete basis for assessment of *in vivo* effects. Animal studies are complicated by differences in the effect of bacterial endotoxin on different animal species and in different models with the same species. While humans are exquisitely sensitive to endotoxin, the responses of other animals vary significantly. For example, mice and rats are far more resistant to endotoxin on a weight basis than are rabbits and dogs; a life-threatening dose in rabbits would produce minimal effects in mice. Moreover, the types of effects noted are quite variable. Dogs display intestinal hemorrhages following sublethal but shock-producing doses of endotoxin while other commonly used laboratory animals do not. Mice housed at usual room temperature become hypothermic after injection of endotoxin but develop fever when housed at 30°C. See, *e.g.*, page xx in the Introduction in *Cellular Biology of Endotoxin*, L. Berry ed., Volume 3 in the series *Handbook of Endotoxin* (R. Proctor, series ed.) Elsevier, Amsterdam, 1985.

[0005] Of interest to the background of the invention are numerous reports concerning the *in vivo* effects of administration of endotoxin to healthy human volunteers. Martich *et al., Immunobiol.*, **187**:403-416 (1993) provides a current and detailed review of the literature addressing the effects on circulatory system constituents brought about by experimental endotoxemia in otherwise healthy humans. Noting that the responses initiated by endotoxin in humans are common to the acute inflammatory response that is part of the host reaction to tissue injury or infection, the authors maintain that administration of endotoxin serves as a unique means of evaluating inflammatory responses as well as responses specific to endotoxin. The authors also note that, while administration of endotoxin to healthy humans is not a precise model for the entirety of host responses in septic shock, it does allow investigation of the initial host inflammatory response to bacterial endotoxin.

[0006] Martich *et al.* note that intravenous administration of endotoxin is uniformly accompanied by a febrile response and various constitutional changes (myalgia and the like) which are attenuated by ibuprofen but not by the phosphodiesterase inhibitor, pentoxifylline. Cardiovascular responses qualitatively similar to those observed in clinical sepsis are observed in experimental endotoxemia in humans. Characteristic increases are observed in circulating cytokines such as tumor necrosis factor α (TNF), interleukin 6 (IL-6); interleukin 1β (IL-1β), interleukin 8 (IL-8), and granulocyte colony stimulating factor (GCSF). Inhibitory soluble receptors of TNF were also noted to rise in a characteristic pattern following increases in levels of circulating TNF. The studies reported on in Martich *et al.* provided observations that ibuprofen increased levels of circulating TNF and IL-6 in experimental endotoxemia and that pentoxifylline decreased circulating TNF, but not circulating IL-6.

[0007] Human experimental endotoxemia was noted to give rise to humoral inflammatory responses similar to those observed in sepsis. The fibrinolytic system is activated and levels of tissue plasminogen activator (tPA) in circulation rise, accompanied by increases in α 2-plasmin inhibitor-plasmin complexes (PAP), confirming activation of plasminogen by tPA. Endotoxin administration to humans has been observed to prompt transitory leukopenia followed by rapid

leukocytosis. Neutrophil degranulation occurs with attendant release of elastase (measured as elastase/α1-antitrypsin (EAA) complexes) and lactoferrin into circulation.

[0008] Martich *et al.* conclude that endotoxin administration to humans represents an important model of acute inflammation which reproduces many of the inflammatory events that occur during sepsis and septic shock and provides a unique means of studying host responses to an important bacterial product.

[0009] Following publication of the Martich *et al.* review article, the same research group reported on a study of experimental endotoxemia wherein an attempt was made to ascertain whether endotoxin administration into circulation could give rise to increased cytokine levels in the lung as measured by broncheoalveolar lavage (BAL). Boujoukos *et al., J. Appl. Physiol.*, **74(6)**:3027-3033 (1993). Even when ibuprofen was co-administered to enhance endotoxin mediated levels of circulating TNF and IL-6 in humans, no increases in TNF, IL-6 or IL-8 levels were observed in BAL fluid, suggesting that cytokine responses to endotoxin in circulation were compartmentalized and did not directly involve lung tissue endothelia.

[0010] Studies of the cardiovascular disturbances in septic shock have established that shock is usually characterized by a high cardiac index (CI) and a low systemic vascular resistance index (SVRI). [Parker *et al., Crit. Care. Med.*, **15**: 923-929 (1987); Rackow *et al., Circ. Shock*, **22**:11-22 (1987); and Parker *et al., Ann. Intern. Med.*, **100**:483-490 (1984).] Of additional interest to the background of the invention are studies of experimental endotoxemia in humans which have demonstrated depression of myocardial contractility and diastolic dysfunction. [Suffredini *et al., N. Eng. J. Med.*, **321**:280-287 (1989).]

[0011] Bactericidal/Permeability-Increasing protein (BPI) is a protein isolated from the granules of mammalian polymorphonuclear neutrophils (PMNs), which are blood cells essential in the defense against invading microorganisms. Human BPI protein isolated from PMNs by acid extraction combined with either ion exchange chromatography [Elsbach, *J. Biol. Chem.*, **254**:11000 (1979)] or *E. coli* affinity chromatography [Weiss, *et al., Blood*, **69**:652 (1987)] has optimal bactericidal activity against a broad spectrum of gram-negative bacteria. The molecular weight of human BPI is approximately 55,000 daltons (55 kD). The amino acid sequence of the entire human BPI protein, as well as the DNA encoding the protein, have been elucidated in Figure 1 of Gray *et al., J. Biol. Chem.*, **264**:9505 (1989), incorporated herein by reference.

[0012] The bactericidal effect of BPI has been shown in the scientific literature published to date to be highly specific to sensitive gram-negative species, with toxicity generally lacking for other microorganisms and for eukaryotic cells. The precise mechanism by which BPI kills bacteria is not yet completely elucidated, but it is known that BPI must first attach to the surface of susceptible gram-negative bacteria. This initial binding of BPI to the bacteria involves electrostatic and hydrophobic interactions between the basic BPI protein and negatively charged sites on endotoxin. BPI binds to lipid A, the most toxic and most biologically active component of endotoxins.

[0013] In susceptible bacteria, BPI binding is thought to disrupt lipopolysaccharide (LPS) structure, leading to activation of bacterial enzymes that degrade phospholipids and peptidoglycans, altering the permeability of the cell's outer membrane, and initiating events that ultimately lead to cell death. [Elsbach and Weiss, *Inflammation: Basic Principles and Clinical Correlates*, eds. Gallin *et al.*, Chapter 30, Reven Press, Ltd. (1992)]. BPI is thought to act in two stages. The first is a sublethal stage that is characterized by immediate growth arrest, permeabilization of the outer membrane and selective activation of bacterial enzymes that hydrolyze phospholipids and peptidoglycan. Bacteria at this stage can be rescued by growth in serum albumin supplemented media. The second stage, defined by growth inhibition that cannot be reversed by serum albumin, occurs after prolonged exposure of the bacteria to BPI and is characterized by extensive physiologic and structural changes, including penetration of the cytoplasmic membrane.

[0014] Permeabilization of the bacterial cell envelope to hydrophobic probes such as actinomycin D is rapid and depends upon initial binding of BPI to endotoxin, leading to organizational changes which probably result from binding to the anionic groups in the KDO region of endotoxin, which normally stabilize the outer membrane through binding of Mg^{++} and Ca^{++}. Binding of BPI and subsequent bacterial killing depends, at least in part, upon the endotoxin polysaccharide chain length, with long O chain bearing organisms being more resistant to BPI bactericidal effects than short, "rough" organisms [Weiss *et al., J. Clin. Invest.*, **65**: 619-628 (1980)]. This first stage of BPI action is reversible upon dissociation of the BPI from its binding site. This process requires synthesis of new LPS and the presence of divalent cations [Weiss *et al., J. Immunol.*, **132**: 3109-3115 (1984)]. Loss of bacterial viability, however, is not reversed by processes which restore the outer membrane integrity, suggesting that the bactericidal action is mediated by additional lesions induced in the target organism and which may be situated at the cytoplasmic membrane [Mannion *et al., J. Clin. Invest.*, **86**: 631-641 (1990)]. Specific investigation of this possibility has shown that on a molar basis BPI is at least as inhibitory of cytoplasmic membrane vesicle function as polymyxin B [In't Veld *et al., Infection and Immunity*, **56**: 1203-1208 (1988)] but the exact mechanism has not yet been elucidated.

[0015] A proteolytic fragment corresponding to the N-terminal portion of human BPI holoprotein possesses essentialy all the bactericidal efficacy of the naturally-derived 55 kD human holoprotein. [Ooi *et al., J. Bio. Chem.*, **262**: 14891-14894 (1987)]. In contrast to the N-terminal portion, the C-terminal region of the isolated human BPI protein displays only slightly detectable anti-bacterial activity. [Ooi *et al., J. Exp. Med.*, **174**:649 (1991).] A BPI N-terminal

fragment, comprising approximately the first 199 amino acid residues of the human BPI holoprotein and referred to as "$rBPI_{23}$," has been produced by recombinant means as a 23 kD protein. [Gazzano-Santoro *et al., Infect. Immun.*, **60**: 4754-4761 (1992).]

[0016] Of additional interest to the present application are the disclosures of references which relate to the potentiation of BPI bactericidal activity by 15 kD proteins derived from the granules of rabbit PMNs designated p15. Ooi *et al., J. Biol. Chem.*, **265**: 15956 (1990), disclose two related 15 kD proteins derived from rabbit PMN granules which potentiate the first sublethal stage of BPI antibacterial activity but have an inhibitory effect on the second lethal stage of BPI antibacterial activity. Levy *et al., J. Biol. Chem.*, **268**: 6058-6063 (1993), disclose the sequences of cDNAs encoding the two rabbit proteins and report that the protein with the stronger potentiating effect reduces the required dose of BPI for the early bacteriostatic effect by about 20-fold.

[0017] Of particular interest to the background of the present invention are reports of interaction between bacterial endotoxin and BPI protein products in various *in vitro* and non-human *in vivo* assay systems. As one example, Leach *et al.*, Keystone Symposia "Recognition of Endotoxin in Biologic Systems", Lake Tahoe, CA, March 1-7, 1992 (Abstract) reported that $rBPI_{23}$ (as described in Gazzano-Santoro *et al., supra)* prevented lethal endotoxemia in actinomycin D-sensitized CD-1 mice challenged with E. *coli* 011:B4 LPS. In additional studies Kohn *et al., J. Infectious Diseases, 168*: 1307-1310 (1993) demonstrated that $rBPI_{23}$ not only protected actinomycin-D sensitized mice in a dose-dependent manner from the lethal effects of LPS challenge but also attenuated the LPS-induced elevation of TNF and IL-1 in serum. Ammons *et al.*, [*Circulatory Shock, 41:* 176-184 (1993)] demonstrated in a rat endotoxemia model that $rBPI_{23}$ produced a dose-dependent inhibition of hemodynamic changes associated with endotoxemia. Kelly *et al., Surgery, 114:* 140-146 (1993) showed that $rBPI_{23}$ conferred significantly greater protection from death than an antiendotoxin monoclonal antibody (E5) in mice innoculated intratracheally with a lethal dose of *E. coli.* Kung, *et al.*, International Conference on Endotoxin Amsterdam IV, August 17-20 (1993) (Abstract) disclosed the efficacy of $rBPI_{23}$ in several animal models including live bacterial challenge and endotoxemia models.

[0018] M.N. Marra and R.W. Scott and co-workers have addressed endotoxin interactions with BPI protein products in U.S. Letters Patent Nos. 5,089,274 and 5,171,739, in published PCT Application WO 92/03535 and in Marra *et al., J. Immunol.*, **144**:662-665 (1990) and Marra *et al., J. Immunol.*, **148**:532-537 (1992). *In vitro* and non-human *in vivo* experimental procedures reported in these documents include positive assessments of the ability of BPI-containing granulocyte extracts, highly purified granulocytic BPI and recombinant BPI to inhibit endotoxin stimulation of cultures of human adherent mononuclear cells to produce tumor necrosis factor α (TNF) when endotoxin is pre-incubated with the BPI product. Pre-incubation of endotoxin with BPI protein products was also shown to diminish the capacity of endotoxin to stimulate (upregulate) neutrophil cell surface expression of receptors for the complement system components C3b and C3bi *in vitro.* However, neither of these complement system components is known to have been demonstrated to be present in increased amounts in circulation as a result of the presence of endotoxin in human circulation. The experimental studies reported in these documents included in *vivo* assessments of endotoxin interaction with BPI protein products in test subject mice and rats. In one series of experiments, BPI was noted to inhibit stimulation of lung cell production of TNF (measured on the basis of cytotoxicity to fibrosarcoma cells of broncheoalveolar lavage fluids) in mice challenged by intranasal administration of endotoxin. In another series of experiments, administration of BPI was noted to protect mice and rats from lethal challenge with various bacterial endotoxin preparations and live *Pseudomonas* and binding of BPI to endotoxin was noted to diminish pyrogenicity in rabbits. As noted above, however, Boujoukas *et al., J. Appl. Physiol.*, **74(6)**:3027-3033 (1993) have demonstrated that, while administration of endotoxin to human circulation resulted in increased levels of circulating TNF, IL-6 and IL-8, no increases in these substances could be detected in broncheoalveolar lavage fluids of the human subjects.

[0019] Since the filing of parent U.S. Patent Application Serial No. 08/188,221 on January 24, 1994, additional studies of *in vitro* and *in vivo* effects of BPI have been published. Fisher *et al., Critical Care Med.*, **22(4)**:553-558 (1994) addressed studies in mice, rats and rabbits and concluded that, "The exciting possibility that bactericidal/permeability-increasing protein may be a specific therapeutic agent to enhance the natural negative feedback mechanisms for regulating endotoxin in humans is worth investigation." Marra *et al., Critical Care Med.*, **22(4)**:559-565 (1994) addressed studies in mice and concluded that, "The potent endotoxin-binding and -neutralizing properties of bactericidal/permeability-increasing protein indicate that it might be useful in the treatment of endotoxin-related disorders in humans."

[0020] Thus, while BPI protein products have been established to have potentially beneficial interactions with endotoxin in a variety of *in vitro* and non-human *in vivo* model systems, nothing is known concerning effects of these products in humans actually exposed to bacterial endotoxin in circulation as a result of, e.g., Gram negative bacterial infection, treatment with antibiotics, accidental injection with endotoxin-contaminated fluids, translocation of endotoxin from the gut, and the like.

SUMMARY OF THE INVENTION

[0021] The present invention provides novel methods for treatment of humans exposed to bacterial endotoxin in

circulation involving the administration of BPI protein products to provide serologically, hematologically and hemodynamically verifiable alleviation of endotoxin effects. The invention thus addresses the use of BPI protein products in the manufacture of pharmaceutical compositions for the treatment of humans exposed to bacterial endotoxin in circulation.

[0022] According to one aspect of the invention, BPI protein products such as $rBPI_{23}$ are administered to humans in amounts sufficient to provide serologically, hematologically and hemodynamically verifiable alleviation of endotoxin mediated effects including, but not limited to: increases in circulating tumor necrosis factor (TNF), soluble TNF receptors p55 and p75 [sTNFr (p55) and sTNFr (p75)], interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10) and increased neutrophil degranulation characterized by increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes (EAA); increases in circulating tissue plasminogen activator antigen (tPA Ag), tissue plasminogen activator activity (tPA act), and α2-plasmin inhibitor-plasmin (PAP) complexes, plasminogen activator inhibitor antigen (PAI Ag) and urokinase type plasminogen activator (uPA); decreases in lymphocytes; increases in thrombin/antithrombin III (TAT) complexes; and decreases in systemic vascular resistance index (SVRI) and increases in cardiac index (CI).

[0023] According to another aspect of the invention BPI protein products are conjointly administered to human patients receiving antibiotic therapy to ameliorate, in a serologically, hematologically and hemodynamically verifiable manner, the effects of endotoxin release into circulation normally attending antibiotic mediated cytolysis or breakdown of bacteria.

[0024] In its presently preferred form, BPI protein products are administered according to the invention in dosage amounts of about 0.1 to about 10 mg/kg of body weight by parenteral, *e.g.*, intravenous, routes in single and multiple dosage formats or by continuous infusion. Oral and aerosolized administration is also within the contemplation of the invention.

[0025] The present invention provides a use of a BPI protein product for the manufacture of a medicament for treatment of humans exposed to bacterial endotoxin in circulation. This aspect of the invention contemplates use of a BPI protein product in the manufacture of such medicaments in an amount effective to alleviate endotoxin mediated increase in circulating tumor necrosis factor and interleukin 6; in an amount effective to alleviate endotoxin mediated increase in circulating interleukin 8 and in neutrophil degranulation as characterized by increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes; in an amount effective to alleviate endotoxin mediated changes in numbers of circulating lymphocytes; in an amount effective to alleviate endotoxin mediated increase in circulating tissue plasminogen activator and tissue plasminogen activator activity; and in an amount effective to alleviate endotoxin-mediated decreases in systemic vascular resistance index. This aspect of the invention further contemplates use of a BPI protein product in combination with bacterial antibiotics in the manufacture of such medicaments.

[0026] Other aspects and advantages of the present human treatment method inventions will be apparent to those skilled in the art upon consideration of the following detailed description of presently preferred embodiments thereof, reference being made to the drawing wherein data is presented for human patients exposed to bacterial endotoxin and either placebo-treated (open circles) or treated with a BPI protein product according to the invention (filled circles). In Figures 2-19, the data points for the placebo-treated patients (open circles) are offset on the time axis slightly to the right of the corresponding data points for the BPI-treated patients (filled circles) for ease of visual comparison.

FIG. 1 is a graphic representation of the results of serological analysis for bacterial endotoxin;
FIG. 2 is a graphic representation of the results of serological analysis for TNF;
FIG. 3 is a graphic representation of the results of serological analysis for soluble TNF p55 receptor;
FIG. 4 is a graphic representation of the results of serological analysis for soluble TNF p75 receptor;
FIG. 5 is a graphic representation of the results of serological analysis for IL-6;
FIG. 6 is a graphic representation of the results of serological analysis for IL-8;
FIG. 7 is a graphic representation of the results of serological analysis for IL-10;
FIG. 8 is a graphic representation of the results of serological analysis for lactoferrin;
FIG. 9 is a graphic representation of the results of serological analysis for elastase/α 1 antitrypsin complexes;
FIG. 10 is a graphic representation of the results of hematological analysis for neutrophils;
FIG. 11 is a graphic representation of the results of hematological analysis for lymphocytes;
FIG. 12 is a graphic representation of the results of serological analysis for tissue plasminogen activator antigen;
FIG. 13 is a graphic representation of the results of serological analysis for tissue plasminogen activator activity;
FIG. 14 is a graphic representation of the results of serological analysis for plasminogen activator inhibitor antigen;
FIG. 15 is a graphic representation of the results of serological analysis for α2-plasmin inhibitor-plasmin complexes;
FIG. 16 is a graphic representation of the results of serological analysis for urokinase type plasminogen activator;
FIG. 17 is a graphic representation of the results of serological analysis for thrombin/antithrombin III (TAT) complexes.
FIG. 18 is a graphic representation of the results of analysis of systematic vascular resistance index (SVRI); and
FIG. 19 is a graphic representation of the results of analysis of cardiac index (CI).

DETAILED DESCRIPTION

[0027] As used herein, "BPI protein product" includes naturally and recombinantly produced BPI protein; natural, synthetic, and recombinant biologically active polypeptide fragments of BPI protein; biologically active polypeptide variants of BPI protein or fragments thereof, including hybrid fusion proteins and dimers; and biologically active polypeptide analogs of BPI protein or fragments or variants thereof, including cysteine-substituted analogs. The BPI protein products administered according to this invention may be generated and/or isolated by any means known in the art. U.S. Patent No. 5,198,541, the disclosure of which is hereby incorporated by reference, discloses recombinant genes encoding and methods for expression of BPI proteins including recombinant BPI holoprotein, referred to as $rBPI_{50}$ and recombinant fragments of BPI. Co-owned, copending U.S. Patent Application Ser. No. 07/885,501 and a continuation-in-part thereof, U.S. Patent Application Ser. No. 08/072,063 filed May 19, 1993 which are hereby incorporated by reference, disclose novel methods for the purification of recombinant BPI protein products expressed in and secreted from genetically transformed mammalian host cells in culture and discloses how one may produce large quantities of recombinant BPI products suitable for incorporation into stable, homogeneous pharmaceutical preparations.

[0028] Biologically active fragments of BPI (BPI fragments) include biologically active molecules that have the same amino acid sequence as a natural human BPI holoprotein, except that the fragment molecule lacks amino-terminal amino acids, internal amino acids, and/or carboxy-terminal amino acids of the holoprotein. Nonlimiting examples of such fragments include an N-terminal fragment of natural human BPI of approximately 25 kD, described in Ooi et al., *J. Exp. Med.*, 174:649 (1991), and the recombinant expression product of DNA encoding N-terminal amino acids from 1 to about 193 or 199 of natural human BPI, described in Gazzano-Santoro et al., *Infect. Immun. 60*:4754-4761 (1992), and referred to as $rBPI_{23}$. In that publication, an expression vector was used as a source of DNA encoding a recombinant expression product ($rBPI_{23}$) having the 31-residue signal sequence and the first 199 amino acids of the N-terminus of the mature human BPI, as set out in Figure 1 of Gray et al., *supra,* except that valine at position 151 is specified by GTG rather than GTC and residue 185 is glutamic acid (specified by GAG) rather than lysine (specified by AAG). Recombinant holoprotein (rBPI) has also been produced having the sequence (SEQ. ID NOS: 1 and 2) set out in Figure 1 of Gray et al., *supra,* with the exceptions noted for $rBPI_{23}$ and with the exception that residue 417 is alanine (specified by GCT) rather than valine (specified by GTT). Other examples include dimeric forms of BPI fragments, as described in co-owned and co-pending U.S. Patent Application Serial No. 08/212,132, filed March 11, 1994, the disclosure of *which is hereby incorporated by reference.*

[0029] Biologically active variants of BPI (BPI variants) include but are not limited to recombinant hybrid fusion proteins, comprising BPI holoprotein or a biologically active fragment thereof and at least a portion of at least one other polypeptide, and dimeric forms of BPI variants. Examples of such hybrid fusion proteins and dimeric forms are described by Theofan et al. in co-owned, copending U.S. Patent Application Serial No. 07/885,911, and a continuation-in-part application thereof U.S. Patent Application Serial No. 08/064,693 filed May 19, 1993 which are incorporated herein by reference in their entirety and include hybrid fusion proteins comprising, at the amino-terminal end, a BPI protein or a biologically active fragment thereof and, at the carboxy-terminal end, at least one constant domain of an immunoglobulin heavy chain or allelic variant thereof.

[0030] Biologically active analogs of BPI (BPI analogs) include but are not limited to BPI protein products wherein one or more amino acid residue has been replaced by a different amino acid. For example, co-owned, copending U. S. Patent Application Ser. No. 08/013,801 (Theofan et al., "Stable Bactericidal/Permeability-Increasing Protein Products and Pharmaceutical Compositions Containing the Same," filed February 2, 1993), the disclosure of which is incorporated herein by reference, discloses polypeptide analogs of BPI and BPI fragments wherein a cysteine residue is replaced by a different amino acid. A preferred BPI protein product described by this application is the expression product of DNA encoding from amino acid 1 to approximately 193 or 199 of the N-terminal amino acids of BPI holoprotein, but wherein the cysteine at residue number 132 is substituted with alanine and is designated $rBPI_{21}\Delta cys$ or $rBPI_{21}$.

[0031] Other BPI protein products useful according to the methods of the invention are peptides derived from or based on BPI produced by recombinant or synthetic means (BPI-derived peptides), such as those described in co-owned and copending U.S. Patent Application Serial No. 08/209,762, filed March 11, 1994, which is a continuation-in-part of U.S. Patent Application Serial No. 08/183,222, filed January 14, 1994, which is a continuation-in-part of U.S. Patent Application Ser. No. 08/093,202, filed July 15, 1993), which is a continuation-in-part of U.S. Patent Application Ser. No. 08/030,644, filed March 12, 1993, the disclosures of which are hereby incorporated by reference. Other useful BPI protein products include peptides based on or derived from BPI which are described in co-owned and co-pending U.S. Patent Application Serial No. 08/274,299, filed July 11, 1994, by Horwitz *et al.* and U.S. Patent Application Serial No. 08/273,540, filed July 11, 1994, by Little *et al.*

[0032] Presently preferred BPI protein products include recombinantly-produced N-terminal fragments of BPI, especially those having a molecular weight of approximately between 21 to 25 kD such as $rBPI_{23}$ or $rBPI_{21}$ and dimeric forms of these N-terminal fragments. Additionally, preferred BPI protein products include $rBPI_{50}$ and BPI-derived pep-

tides.

[0033] It is also contemplated that the BPI protein product be administered with other products that potentiate the activity of BPI protein products. For example, serum complement potentiates the gram-negative bactericidal activity of BPI protein products; the combination of BPI protein product and serum complement provides synergistic bactericidal/growth inhibitory effects. See, e.g., Ooi *et al. J. Biol. Chem., 265: 15956* (1990) and Levy *et al. J. Biol. Chem., 268*: 6038-6083 (1993) which address naturally-occurring 15 kD proteins potentiating BPI antibacterial activity. See also co-owned, co-pending U.S. Patent Application Serial No. 08/093,201, filed July 14, 1993, and continuation-in-part, U.S. Patent Application Serial No. 08/274,373, filed July 11, 1994 which describes methods for potentiating gram-negative bactericidal activity of BPI protein products by administering lipopolysaccharide binding protein (LBP) and LBP protein products. The disclosures of these applications are incorporated by reference herein. LBP protein derivatives and derivative hybrids which lack CD-14 immunostimulatory properties are described in co-owned, co-pending U.S. Patent Application Serial No. 08/261,660, filed June 17, 1994 as a continuation-in-part of U.S. Patent Application Serial No. 08/079,510, filed June 17, 1993, the disclosures of which are incorporated by reference herein. See also, the disclosure of immunoassays for the quantification of LBP disclosed in co-owned and co-pending U.S. Patent Application Serial No. filed January 24, 1995 (Attorney Docket No. 27129/32407) as a continuation-in-part of U.S. Patent Application Serial No. 08/186,811 filed January 24, 1994, the disclosures of which are incorporated by reference herein.

[0034] Practice of the methods of the present invention is illustrated in the following examples wherein: Example 1 presents the protocol for a controlled, double-blind crossover study of the effects of a BPI protein product on human volunteers challenged with bacterial endotoxin; Example 2 addresses serological analysis for cytokines and cytokine-related substances; Example 3 addresses lactose and glucose serological analysis; Example 4 addresses total and differential leukocyte analysis and leukocyte activation analysis; Example 5 addresses serological analysis of various coagulation and fibrinolysis parameters; and Example 6 addresses analysis of hemodynamic parameters, specifically left ventricular function.

EXAMPLE 1

[0035] A controlled, double-blind crossover study was designed to investigate the effects of BPI protein products (represented by the recombinant-produced amino terminal fragment referred to as $rBPI_{23}$) in humans rendered endotoxemic by intravenous infusion of bacterial endotoxin.

[0036] Eight healthy male volunteers participated in the study. Each had an unremarkable medical history, a normal physical exam and essentially normal results in routine laboratory tests. EKG, chest X-ray and echocardiogram results were within the normal range for all volunteers. Participation in the study was excluded if any infectious disease had occurred within one month prior to the study, if any acute illness was reported one week prior to the study or if any medication was taken within two weeks prior to the study. Written informed consent was obtained from all participants and the study was approved by an ethical review board. The study had a cross over design in which the subjects were challenged with endotoxin on two occasions, separated by a 6 week wash out period. Placebo or $rBPI_{23}$ was administered concurrently with endotoxin at either of the two occasions, so that each volunteer served as his own control. The treatment sequence ($rBPI_{23}$ followed by placebo, placebo followed by $rBPI_{23}$) was determined by randomization in a 1:1 ratio to the two sequences. Thus four volunteers were treated with $rBPI_{23}$ during the first cycle of endotoxin challenge, and four during the second cycle.

[0037] The study drug, $rBPI_{23}$, was prepared according to the method of Gazzano-Santoro *et al., supra,* and was supplied as a clear, colorless, sterile, non-pyrogenic solution in 10 ml single use glass vials at a concentration of 1 mg/ml in a buffer of 20 mmol/l sodium citrate, 0.15 M sodium chloride, 0.1% poloxamer 188, 0.002% polysorbate 80, pH 5.0, containing no preservative. The placebo solution was supplied as a clear, colorless, sterile, non-pyrogenic solution in 10 ml single use glass vials. This solution contained 0.2 mg/ml human albumin in a buffer of 20 mmol/l sodium citrate, 0.15 mol/l sodium chloride, pH 5.0, containing no preservative. A treatment kit for each subject was coded according to the randomization schedule.

[0038] The endotoxin preparation used was FDA lot EC-5 (*Escherichia coli*), kindly provided by Dr. D. Hochstein (Bureau of Biologics, U.S. Food and Drug Administration, Bethesda, MD). Before injection, the endotoxin preparation was reconstituted according to FDA directions, warmed to 37°C, vigorously shaken for 30 minutes, and diluted to the appropriate concentration in endotoxin-free saline. $rBPI_{23}$ or placebo were administered in exactly five minutes, in a forearm vein. Endotoxin was injected intravenously in one minute, in a forearm vein of the opposite arm, at a dose of 40 EU/kg during the third minute of the test drug infusion. Blood pressure and heart rate were monitored at 15 minute intervals using a dinamap device (Critikon, Tampa Florida).

[0039] Blood was obtained through an indwelling intravenous catheter, at the following time points: -30, 3, 5, 7, 12, 20, 30, 35 minutes, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 hours. Blood was collected in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose for the BPI determination, in pyrogen-free plastic tubes (Falcon 2063, Oxnard, CA) containing pyrogen-free heparin (Thromboliquine® Organon, Oss, Netherlands, final concentration 30 U/

ml) for the endotoxin test, in tubes containing K_3-EDTA for leukocyte counts, and in siliconized Vacutainer tubes (Becton Dickinson) to which soybean trypsin inhibitor was added for the elastase/α_1-antitrypsin complex, lactoferrin and C3a-desarg assays. Serum was prepared for all other tests by centrifugation of clotted blood for 20 minutes at 2000 x g.

[0040] For each experimental assessment made, data were analyzed to determine both mean and median values and the associated standard errors. Results were plotted as a function of the relevant time period. Statistical significance of treatment difference was assessed for median values using the Wilcoxon signed rank test [Lehmann, Nonparametrics-Statistical Methods Based on Ranks, (Holden-Day, Inc., San Francisco, CA 1975)] on the percent change in trapezoidal area under the curve (AUC) for BPI protein product treatment relative to the AUC on placebo treatment for each volunteer. Percent change in AUC for $rBPI_{23}$ treatment relative to placebo was defined as $100 \times (AUC_{BPI}-AUC_{placebo})/(AUC_{placebo})$. A negative percent change implies a reduction in AUC for $rBPI_{23}$ treatment relative to placebo treatment. Statistical significance was assessed separately in each functional group of assays so that the type 1 error rate for each group of parameters was maintained at α=0.05. The Hochberg method [Hochberg, *Biometrika,* **75**:800-802 (1988)], an improved Bonferroni procedure [Miller, Simultaneous Statistical Inference (Springer-Verlag, New York, New York, second ed., 1981)] for multiple significance testing, was used to determine statistical significance within each group of parameters. Tests for carryover and period effects were performed according to the nonparametric method described by Koch, *Biometrics,* **28**:577-584 (1972). Where period effects were observed by this method, treatment differences were further explored by performing the test for treatment effect in the presence of period effects as described in Koch, *supra.*

[0041] Infusion of $rBPI_{23}$ concomitantly with endotoxin resulted in a transient (average of 15 minutes) flush in six of the eight volunteers, but caused no other signs. Following infusion of endotoxin, oral temperature rose similarly after placebo or BPI infusion: following infusion of endotoxin and placebo, from 36.0 ± 0.2°C to 37.8 ± 0.1°C; with $rBPI_{23}$ infusion, from 35.6 ± 0.2°C to 37.5 ± 0.3°C. The mean arterial blood pressure decreased in both study periods, and was not influenced by the BPI infusion. No safety related EKG changes were noted in any of the volunteers. Volunteers suffered from clinical symptoms such as headache, myalgia, chills and nausea in both treatment regimens. All volunteers were completely recovered at 24 hours following the start of the infusion, and renal, liver and hematological parameters were within the normal range at this time point.

[0042] Vital sign parameters including systolic blood pressure (SBP), diastolic blood pressure (DPP), mean arterial pressure (MAP), pulse, respiration rate and temperature were assessed. Table 1, below, sets out the results of statistical analysis of vital signs and reveals that there was no statistically significant difference in values for the $rBPI_{23}$-treated patients relative to placebo patients.

TABLE 1

VITAL SIGNS				
Parameter	**AUC hour**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
DBP	0-10	-1%	.95	NS
MAP	0-10	+1%	.74	NS
Resp Rate	0-10	-6%	.64	NS
SBP	0-10	+3%	.55	NS
Temp	0-10	-4%	.1094	NS
Pulse	0-10	-21%	.0391	NS

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS =nonsignificant).

[0043] Endotoxin assays were performed as described in van Deventer *et al., Blood,* **76(12)**:2520-2526 (1990). Results are graphically represented in FIG. 1 wherein mean values ± standard error of the mean for placebo-treated patients are represented by open circles and values for BPI treated patients are represented by filled circles. Over the period of zero time to 20 minutes, endotoxin levels were significantly higher following placebo treatment in comparison to $rBPI_{23}$ treatment (Median percent change in AUC = 98%; p-value = .0156).

EXAMPLE 2

Cytokine and Cytokine Related Proteins

[0044] Serum levels of TNF were determined by immunoradiometric assay IRMA Medgenix, Fleurus, Belgium). Briefly noted, polypropylene tubes were coated with a combination of monoclonal antibodies to recombinant TNF that rec-

ognize distinct epitopes of TNF. The tubes were incubated overnight with a mixture of the sample to be tested and anti-TNF antibody labeled with ^{125}I. After decantation, the bound fraction was counted in a gamma-counter, and the level of TNF was expressed in pg/ml in relation to a standard binding curve for recombinant TNF.

[0045] The serum concentrations of IL-1β, IL-8 and IL-10 were performed by a commercial ELISA kit according to the instructions of the manufacturer (Medgenix, Fleurus, Belgium). Serum concentration of IL-6 was also determined by ELISA according to manufacturer's instructions. [Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) Amsterdam, Netherlands].

[0046] Soluble TNF receptor p55 and p75 concentrations were measured by specific enzyme linked immunological assays as described in Van der Poll et al., *J. Infect. Dis.*, **168**:955-960 (1993). Briefly, microtiter plates (Maxisorp, Nunc, Denmark) were coated overnight at room temperature with TNF-binding non-inhibitory monoclonal antibodies against TNFR-p55 (clone htr-20) or TNFR-p75 (clone utr-4), kindly provided by Dr. H. Gallati, Hoffmann LaRoche Ltd., Basel, Switzerland. Subsequently, the coated wells were washed and the remaining protein-binding capacity of the wells was saturated with 1% BSA in 200 mM Tris/HCl, pH 7.5, 0.02% kathon (Hoffmann LaRoche Ltd., Basel, Switzerland). After discarding the storage buffer, samples diluted 1:5 in 0.1 M Tween 20, pH 7.25, containing 10% fetal bovine serum, 0.1% phenol, 0.1% Tween 20, 0.02% kathon were added to the wells. Standard curves were constructed with recombinant sTNFR-p55 or sTNFR-p75. Peroxidase-conjugated recombinant human TNF was added and the mixtures were incubated for two days at 4°C. After washing, ortho-phenyldiamine-dihydrochloride substrate was added and incubated for 15-20 min. The reaction was stopped with 1.5 M H_2SO_4 and the absorbance was spectrophotometrically determined at 490 nm.

[0047] Assay results for TNF and the p55 and p75 soluble TNF receptors are graphically presented in FIGS. 2, 3 and 4, respectively. As indicated therein, TNF levels increased following endotoxin/placebo infusion from 0.50 ± 0.38 pg/ml (mean ± SEM) immediately prior to infusion to peak levels of 261.88 ± 60.73 pg/ml at one and one half hours and returned to 3.50 ± 0.91 pg/ml by ten hours. When endotoxin infusion was accompanied by $rBPI_{23}$, however, the TNF peak was blunted (41.13 ± 14.36 pg/ml) and delayed to three hours after infusion. The serum concentrations of both types of TNF receptors were elevated after endotoxin/placebo challenge (TNFR-p55: from 1.21 ± 0.07 ng/mL at time 0 to 3.79 ± 0.29 ng/mL at 3#hr; TNFR-p75: from 3.28 ± 0.24 ng/mL at time 0 to 12.72 ± 1.43 ng/mL at 3 hours) but increased to a lesser extent following endotoxin infusion with $rBPI_{23}$ (TNFR-p55: from 1.12 ± 0.07 ng/mL at time 0 to 2.45 ± 0.16 ng/mL at 4 hours; TNFR-p75: from 3.27 ± 0.29 at time 0 to 7.45 ± 0.71 ng/mL at 6 hours) and peak levels were temporally shifted.

[0048] Post-treatment IL-1 levels were below the assay detection limit during the entire experiment in both treatment periods.

[0049] As indicated in FIG. 5, in endotoxin/placebo treated volunteers IL-6 levels increased from 0.23 ± 0.23 pg/ml (mean ± SEM) prior to infusion to 188.78 ± 79.99 pg/ml at three hours and returned to normal values at 10 hours. After $rBPI_{23}$ treatment, IL-6 rose from 0.06 ± 0.04 pg/ml prior to infusion to reach peak levels of 45.13 ± 23.28 pg/ml at 4 hours and subsequently returned to normal at 10 hours. As indicated in FIG. 6, IL-8 increased from undetectable levels prior to infusion to 69.83 ± 16.72 pg/ml (mean ± SEM) at two hours in endotoxin/placebo treated patients and decreased again to 4.18 ± 2.38 pg/ml at 10 hours following endotoxin infusion. After $rBPI_{23}$ infusion the IL-8 peak occurred later (14.20 ± 10.63 pg/ml at four hours) and IL-8 levels remained undetectable throughout the entire ten hour assessment period in three out of eight volunteers. At 10 hours, no IL-8 was detectable in any volunteer receiving $rBPI_{23}$.

[0050] Serum levels of IL-10 increased in endotoxin challenged volunteers after both placebo and $rBPI_{23}$ treatment. As seen in FIG. 7, peak levels were observed between one and one-half to three hours after infusion. In the placebo group, IL-10 rose from 10.35 ± 4.19 pg/ml (mean ± SEM) prior to infusion to 38.94 ± 10.66 pg/ml (at three hours) and declined to 10.35 ± 4.37 pg/ml (at ten hours). In the $rBPI_{23}$ treated group, IL-10 was increased to a lesser extent; *i.e.*, from an initial level of 8.86 ± 3.35 pg/ml to 21.76 ± 6.89 pg/ml at three hours.

[0051] Table 2, below, sets out the results of statistical analysis of test results for cytokines and cytokine related proteins from time 0 through ten hours.

TABLE 2

CYTOKINES AND CYTOKINE RELATED PROTEINS				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
IL-1	0-10	0%	.25	NS
IL-10	0-10	-38%	.0156	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS = nonsignificant).

TABLE 2 (continued)

CYTOKINES AND CYTOKINE RELATED PROTEINS				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
IL-6	0-10	-79%	.0078	S
IL-8	0-10	-97%	.0078	S
TNF	0-10	-86%	.0078	S
TNF r(p55)	0-10	-40%	.0078	S
1NF r(p75)	0-10	-48%	.0078	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS = nonsignificant).

[0052] The above results establish that treatment of experimental endotoxemia in humans with a BPI protein product resulted in a serologically verifiable and statistically significant modification in cytokine response to the presence of endotoxin in circulation. The severity and temporal setting of TNF production mediated by endotoxin was dramatically altered and was accompanied by corresponding decreases in levels of circulating soluble TNF receptors and a temporal shifting of peak circulating receptor values. In a like manner, the presence of IL-6 in circulation as a consequence of endotoxin in circulation was significantly modified. While one previous study indicated that circulating TNF levels in experimental endotoxemia in humans could be diminished by intervention with pentoxifylline [Zabel *et al., Lancet,* **2**: 1474-1477 (1989)], and ibuprofen has been observed to increase TNF and IL-6 levels following endotoxin administration, no previous study of this type has identified an agent which is effective in reducing TNF and IL-6 responses to endotoxin in humans.

[0053] Reduction in TNF response to endotoxin through administration of $rBPI_{23}$ was accompanied not only by reductions in circulating TNF receptors but also reduction in circulating IL-10, a substance which has been characterized as an anti-inflammatory cytokine. The reduction and shifting in peak IL-8 levels attending is the subject of discussion in Example 4, *infra.*

EXAMPLE 3

Lactate and Glucose Analysis

[0054] Lactate and glucose analyses were performed using standard laboratory procedures. Briefly summarized, serum glucose rose similarly over time in both treatment groups, while in each group there was only a slight rise from baseline levels of lactate.

EXAMPLE 4

Total and Differential Leukocytes and Leukocyte Activation Analysis

[0055] Leukocyte total and differential counts were determined through use of a flow cytometer (Technicon H1 system, Technicon Instruments, Tarrytown, NY). Leukocytes counts decreased from $5.7 \pm 0.7 \times 10^9$/L at time 0 to $4.0 \pm 0.6 \times 10^9$/L at 1.5 hours in the placebo period, and subsequently rose to $10.6 \pm 0.9 \times 10^9$/L at 6 hours. $rBPI_{23}$ blunted both the early leukocyte decrease (leukocyte count at 1.5 hours: $5.9 \pm 0.6 \times 10^9$/L, median change in AUC in the first two hours=+30%, p=0.078), and the subsequent rise in white blood cells ($8.1 \pm 0.8 \times 10^9$/L at 6 hours) so that the median reduction in leukocyte AUC in the first 12 hours was 19% (p=0.039). Marked monocytopenia and lyumphocytopenia developed within the first two hours following infusion of endotoxin, and monocyte and lymphocyte counts returned to baseline levels at 6 and 12 hours, respectively. In the placebo period, monocyte and lymphocyte counts became depressed again at 24 hours following endotoxin challenge (lymphocytes: $1.3 \pm 0.2 \times 10^9$/L; monocytes $0.4 \pm 0.1 \times 10^9$/L). Eosinophil counts decreased following endotoxin infusion with placebo treatment from $0.23 \pm 0.07 \times 10^9$/L at time 0 to $0.04 \pm 0.008 \times 10^9$/L at 8 hours. Neutrophil counts rose in placebo treatment from $3.5 \pm 0.6 \times 10^9$/L at time 0 to a peak level of $9.5 \pm 0.8 \times 10^9$/L at 6 hours, and subsequently decreased to $2.4 \pm 0.3 \times 10^9$/L at 24 hours. As shown in Table 3 below, $rBPI_{23}$ infusion significantly blunted the decrease of lymphycytes (median % change in AUC: +34%; p=0.0078) and blunted the monocytes, eosinophils and neutrophils with median % change on AUC for monocytes of +42% [p=0.023], for eosinophils: +37% [p=0.039] and for neutrophils -26% [p=0.016]. At 24 hours, however, monocyte, lymphocyte and eosinophil counts remained at baseline levels following $rBPI_{23}$ treatment (lym-

phocytes: 1.5 ± 0.2 x 10^9/L; monocytes: 0.5 ± 0.1 x 19^9l; eosinophils: 0.15 ± 0.04 x 10^9/L; neutrophils: 3.2 ± 0.7 x 10^9/L).

[0056] Results for neutrophil and lymphocyte determinations (mean ± SEM) are also set out in graphic form in Figures 10 and 11, respectively. Table 3, below, sets out the results of statistical analysis of total and differential leukocytes from time 0 through 12 hours.

TABLE 3

LEUKOCYTE DIFFERENTIAL				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
Basophils	0-12	+2%	.95	NS
Eosinophils	0-12	+37%	.0391	NS
WBC	0-12	-19%	.0391	NS
Monocytes	0-12	+42%	.0234	NS
Neutrophils	0-12	-26%	.0156	NS
Lymphocytes	0-12	+34%	.0078	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS=nonsignificant).

[0057] Plasma concentrations of elastase/α1-antitrypsin complex (EAA) and lactoferrin were measured by radioimmunoassay according to the method described in Nuijens *et al., J. Lab. Clin. Med.*, **119**:159-168 (1992), and values determined in terms of ng/ml. Complement activation was assessed by measuring the plasma concentrations of C3a-desarg using a radioimmunoassay as described in Hack *et al., J. Immunol. Meth.*, **107**:77-84 (1988) and values were expressed in terms of nmol/l.

[0058] Lactoferrin and EAA analysis results are set out in Table 4 below and mean ± SEM results also graphically represented in Figures 8 and 9, respectively. Elastase-α_1-antitrypsin complexes (EAA) after placebo treatment increased from 62.5 ± 8.5 ng/mL at time 0 to 141 ± 20.9 ng/mL at 4 hours after endotoxin infusion, representing a rise to 2.26 times baseline. In the $rBPI_{23}$ treatment, only a minor increase in elastase/α_1-antitrypsin complex formation was observed (57.5 ± 7.4 ng/mL at time 0 to 96.0 ± 8.7 ng/mL at 6 hours after infusion, a rise to 1.67 times baseline). Lactoferrin concentrations increased to 2.59 times baseline after endotoxin infusion (from 163 ± 22.6 ng/mL at time 0 to 422 ± 58.0 ng/mL at 4 hours). After $rBPI_{23}$ treatment, lactoferrin concentrations increased to only 1.64 times baseline (from 169 ± 27.6 ng/mL at time 0 to 276.6 ± 60.5 ng/mL at 3 hours). The 47% reduction in lactoferrin AUC on $rBPI_{23}$ relative to placebo was significant (p=0.0078) (Table 4). The 36% reduction in EAA AUC was not significant (p=0.15); however, since the test for period effect was significant for this parameter, the treatment effect was further explored by the method described by Koch, *supra*. This analysis suggested a significant treatment effect of $rBPI_{23}$ in lowering EAA AUC (p=0.0304) (Table 4).

TABLE 4

LEUKOCYTE ACTIVATION				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
EAA	0-10	-36%	.0304[c]	S
Lactoferrin	0-10	-47%	.0078	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS=nonsignificant).

[c] Accounting for period effect.

[0059] While no significant differences in C3a-desarg were noted for the two treatment strategies, in BPI-treated volunteers EAA complex formation and lactoferrin assay results were markedly lower than in placebo-treated volunteers. The results observed in the EAA and lactoferrin assays are consistent with the previously noted reduction in IL-8 values in BPI-treated individuals. IL-8 has been implicated in effecting degranulation of neutrophils and rises in lactoferrin and EAA in circulation result from neutrophil degranulation. Thus, the present study reflects the first known instances of intervention in experimental endotoxemia in humans resulting in reduction of circulating levels of endotoxin mediated IL-8 and a coordinated reduction of neutrophil degranulation as assessed by analysis of circulating lactoferrin and EAA.

EXAMPLE 5

Fibrinolysis and Coagulation Parameter Analysis

[0060] Serological analyses were performed to assess circulating levels of D-dimer, prothrombin fragments F1+2 (Frag F1+2), plasminogen activator inhibitor antigen (PAI Ag), plasminogen activator inhibitor activity (PA1 Act), α 2-plasmin inhibitor-plasmin complexes (PAP), protein C activity (Prot. C Act), thrombin-antithrombin III (TAT) complex, α 2-antiplasmin (AAP), plasminogen, tissue plasminogen activator antigen (tPA Ag), tissue plasminogen activator activity (tPA Act) and urokinase type plasminogen activator (uPA). Blood was collected by separate venous punctures from antecubital veins, before and at 1, 1.5, 2, 3, 4, 6 and 10 hours after the start of the endotoxin infusion and through 12 hours for platelets. Blood for AAP, plasminogen, tPA Ag, uPA, PAI Ag, PAI Act, D-dimer, Prot. C Act, Frag F 1+2 and TAT complex measurements (9 volumes) was collected in vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing 3.8% sodium citrate (1 volume). Blood for t-PA Act was collected in Biopool Stabilyte™ tubes (Biopool, Umea, Sweden) containing a low pH citrate anticoagulant which stabilizes t-PA activity by blocking inhibition of t-PA of PAI. For the measurements of PAP complexes, blood was collected in siliconized vacutainer tubes (Becton Dickinson, Plymouth, England) to which EDTA (10mM, final concentration) and Soy Bean Trypsin Inhibitor (Sigma T-9003; final concentration: 0.1 mg/mL) was added to prevent *in vitro* complex formation. Tubes containing K_3-EDTA were used to collect blood for platelet counts. For all measurements except platelet counts, plasma was prepared by centrifuging at 2000xg for 30 minutes at 15°C, after which plasma was frozen at -70°C until batchwise assessment was performed.

[0061] Platelet counts were determined with the use of a flow cytometer (Technicon H1 system, Technicon Instruments, Tarrytown, USA). Plasma levels of TAT complexes and of Frag F 1+2 were measured with ELISA's (Behringwerke AG, Marburg, Germany) [Teitel *et al., Blood,* **59**: 1086-1096 (1982)]. Results are expressed in ng/mL and nmol/L, respectively. Protein C activity was measured by an amidolytic assay, as described in Sturk *et al., Clin. Chim Acta,* **165**: 263-270 (1987). tPA Act was measured by an amidolytic assay [Verheijen *et al., Thromb. Haemostasis,* **48**: 266-269 (1982)]. Briefly, 25µl of plasma was mixed to a final volume of 250 µl with 0.1 M TrisHCl, pH 7.5, 0.1% (v/v) Tween-80, 0.3 mM S-2251 (Kabi Haematology, Mölndal, Sweden), 0.13 M plasminogen and 0.12 mg/ml CNBr fragments of fibrinogen (Kabi Haematology, Mölndal, Sweden). The results are expressed as IU/ml (first International standard of the World Health Organization).

[0062] PAI Act was measured with an amidolytic assay [Chmielewska, *et al., Thromb. Res.,* **31**: 427-436 (1983)] in which the samples were incubated for 10 min at room temperature with an excess of tissue-type plasminogen activator. Part of the t-PA was inhibited by PAI, present in the sample, and formed inactive complexes. Residual t-PA activity was determined by subsequent incubation, with 0.13 µM plasminogen (Kabi Haematology), 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments (t-PA stimulator, Kabi Haematology) and 0.1 mM S-2251 (Kabi Haematology). The amount of plasmin generated in the incubation mixture, determined by the conversion of the chromogenic substrate, was inversely proportional to the PAI Act in the sample. The results of the samples to be tested were related to the results of samples of PAI Act depleted plasma (Kabi Haematology) to which fixed amounts of t-PA were added. Results were expressed in international units (IU), where 1 IU is the amount of PAI Act that inhibits 1 IU t-PA.

[0063] t-PA Ag and PAI Ag were assayed with ELISA's [Holvoet *et al., Thromb. Haemostasis,* **54**: 684 (1985)] (Asserachrom t-PA, Diagnostica Stago, Asnieres-sur-Seine, France and PAI-ELISA kit, monozyme, Charlottenlund, Denmark, respectively). Results are expressed in ng/ml. uPA was measured with a sandwich-ELISA [Binnema *et al., Thromb. Res.,* **43**: 569 (1986)]. The assay measures the urokinase-antigen present in plasma, irrespective of its molecular form i.e. pro-urokinase, active urokinase and urokinase in complex with inhibitors; the results are expressed in ng/ml. Plasminogen activity and AAP were measured by automated amidolytic techniques according to methods described in Peeters *et al., Thromb. Res.,* **28**: 773 (1982). The results are expressed as percentages of normal. D-Dimer was measured with an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnieres-sur Seine, France) [Elms *et al., Thromb. Haemostasis,* **50**: 591 (1983)]. Results are expressed in µg/ml. PAP complexes were measured by a RIA as described in Levi *et al., J. Clin.Invest.* **88**: 1155-1160 (1991). Briefly, specific mAbs, raised against inactivated and complexed α2-antiplasmin were coupled to sepharose beads and incubated with plasma samples. After washing the sepharose with phosphate buffered saline, bound complexes were subsequently incubated with ^{125}I labeled mAbs to plasmin. After another washing procedure, sepharose-bound radioactivity was measured. As standards, serial dilution of plasma in which a maximal amount of PAP complexes was generated by incubation with two chain urokinase (Choay, Paris, France), after pre-incubation of the plasma with methylamine to inactivate α2-macroglobulin were used. The results are expressed as nmol/L.

[0064] Statistical analysis (as described in Example 1) of the results of these analyses are reflected in Tables 5 and 6. The mean ± SEM results of assays of tissue plasminogen activator antigen (t-PA Ag), tissue plasminogen activator activity (t-PA Act), tissue plasminogen activator inhibitor antigen (PAI Ag), α2-plasmin inhibitor-plasmin (PAP) complexes, urokinase type plasminogen activator (uPA) and thrombin/antithrombin III (TAT) complexes are graphically represented in Figures 12, 13, 14, 15,16 and 17, respectively.

TABLE 5

FIBRINOLYSIS				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
α2-antipl	0-10	-3%	.95	NS
d-dimer	0-10	-45%	.31	NS
plasminogen	0-10	-10%	.20	NS
PAI Activity	2-10	-51%	.0304[c]	NS
PAP Complex	0-10	-51%	.0078	S
uPA	0-10	-50%	.0078	S
PAI Ag	2-10	-52%	.0078	S
tPA Ag	1-10	-79%	.0078	S
tPA Activity	1-6	-57%	.0078	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS=nonsignificant).

[c] Accounting for period effect.

TABLE 6

COAGULATION				
Parameter	**AUC hours**	**Median % change in AUC**	**p-value[a]**	**Statistical significance[b]**
Protein C Activity	0-10	-21%	.64	NS
F1 + 2	1-10	-31%	.0391	NS
TAT Complex	1-10	-36%	.0078	S

[a] p-value comparing $rBPI_{23}$ vs. placebo AUC within each subject (Wilcoxon signed rank test).

[b] Statistical significance as determined by the Hochberg method (S=significant, NS=nonsignificant).

[0065] In this study, endotoxin induced the activation of the coagulation system, consistent with Van Deventer *et al., supra,* and Levi *et al., J. Clin. Invest.,* **93**:114 (1994). Specifically, the infusion of endotoxin resulted in 7.3-fold and 7.4-fold increase in plasma levels of TAT-complexes and prothrombin fragment F_{1+2}, respectively. Mean levels of TAT complexes rose from 5.5 ± 1.4 to 40.0 ± 5.3 ng/mL, pleaking at 3 hours after endotoxin administration and therafter gradually decreasing (Figure 17). F_{1+2} plasma levels reached their peaks at four hours after infusion with endotoxin (increasing from 0.78 ± 0.10 nmol/L to 5.77 ± 1.27 nmol/L). No significant changes were seen in plasma levels of protein C activity.

[0066] Infusion with $rBPI_{23}$ resulted in a significant reduction in endotoxin-induced thrombin generation. Specifically, maximal F_{1+2} levels were 3.30 ± 0.39 nmol/L after the administration of endotoxin in combination with $rBPI_{23}$ as compared to after the administration of endotoxin alone; maximal TAT complex levels were 30.8 ± 6.9 ng/mL aftger the combined endotoxin and $rBPI_{23}$ treatment (Figure 17). Treatment with $rBPI_{23}$ had no effect on plasma levels of protein C activity (Table 6). The AUC for TAT complexes was significantly reduced on $rBPI_{23}$ (median reduction 36%, p=0.0078) (Table 6). The AUC for F_{1+2} was also reduced on $rBPI_{23}$; this effect did not reach significance according to the Hochberg, *supra,* method (median reduction 31%, p=0.0391).

[0067] Platelet counts decreased in both treatment groups from a baseline level of 206.5 ± 15.0 x 10^{12} on placebo to 181.6 ± 13.2 x 10^{12} at 4 hours and from 190.4 ± 16.9 x 10^{12} to 174.8 ± 10.7 x 10^{12} at 6 hours following $rBPI_{23}$ treatment (resulting in a 7% reduction of AUC; p=0.19).

[0068] Additionally, in this study, endotoxin induced initial activiation of the fibrinolytic system followed by inhibition, in agreement with Suffredini *et al., N. Engl. J. Med.,* **320**:1165 (1989). Plasminogen activating activity increased from 0.22 ± 0.03 IU/mL to 2.14 ± 0.13 IU/mL, reaching a peak at two hours after the administration of endotoxin alone. This increase in plasminogen activator activity (Figure 13) was paralleled by an increase in t-PA antigen (Figure 12) and u-PA antigen (Figure 16). Peak levels of t-PA antigen at u-PA antigen were 45.6 ± 6.6 ng/mL at 3 hours and 6.0 ± 0.5 ng/mL at 2 hours, respectively. The rise of plasminogen activity was followed by plasmin generation, as reflected by

increasing levels of PAP-complexes (Figure 15) and of D-Dimer, which increased from 4.61 ± 0.38 nmol/L to 12.4 ± 2.1 nmol/mL at 2 hours and from 217 ± 117 ng/mL to 707 ± 120 ng/mL at 10 hours, respectively. Plasma levels of PAI activity and antigen (Figure 14) remained unchanged up to two hours after endotoxin administration. Thereafter a rapid increase was seen up to 34.5 ± 2.6 IU/mL and 225.8 ± 1.3 ng/mL, respectively, both peaking at 4 hours after the infusion. The increase in PAI was followed in an instanteous decrease in t-PA activity and subsequent plasmin generation, as reflected by decreasing levels of PAP-complexes. Since the assays for t-PA and u-PA antigen also measure plasminogen activator in complex with its inhibitor (i.e. PAI), values of these parameters only gradually decreased.

[0069] The simultaneous adminstration of $rBPI_{23}$ and endotoxin resulted in a substantial reduction and delay of endotoxin-induced fibrinolytic activity. Peak levels of plasminogen activator activity (0.61 ± 0.2 IU/mL) and t-PA antigen (16.4 ± 6.7 ng/mL) were reached at 3 and 4 hours, respectively. Upon administration of endotoxin in combination with $rBPI_{23}$, no increase in u-PA antigen was seen. The endotoxin-induced rises of plasma levels of PAP-complexes and D-Dimer were also greatly attenuated by the $rBPI_{23}$ infusion. Peak levels of PAP complexes and D-Dimer were 7.7 ± 1.3 nmol/L at 3 hours and 531 ± 119.5 ng/mL at 10 hours, respectively. The endotoxin-induced increase of PAI activity and antigen was delayed and partly inhibited by the simultaneous administration of $rBPI_{23}$. Peak levels of PAI activity and antigen were reached at 6 hours after infusion of endotoxin and $rBPI_{23}$. The combined administration of endotoxin and $rBPI_{23}$ resulted in a maximal PAI activity level of 22.6 ± 2.9 IU/mL and a maximal PAI antigen level of 122.9 ± 22.0 ng/mL. The AUC for t-PA activity, t-PA antigen, uPA antigen, PAP complexes and PAI antigen were significantly reduced on $rBPI_{23}$ ($p=0.0078$ for each; median AUC reductions varied from 50 to 79%; Table 5). The AUC for PAI activity and D-dimer were reduced on $rBPI_{23}$ treatment although not significantly (median reductions of 51 %, $p=0.0304$ and 45%, $p=0.31$; Table 5). The AUC for plasminogen and $\alpha 2$ antiplasmin were not changed by $rBPI_{23}$ treatment Table 5).

[0070] The placebo/endotoxin results in Figures 12 through 17 confirm a coordinated serological response to endotoxin administration in that shortly after tPA levels rise, there is sharp increase in tPA activity which precipitously drops upon increases in levels of circulating plasminogen activator inhibitor. The results demonstrate coordinated intervention in these related phenomena by treatment with a BPI-protein product. Peak levels in tPA are diminished and temporally shifted and a corresponding drop in tPA activity and circulating plasminogen activator inhibitor level is observed. Activation of plasminogen by tPA, as indicated by levels of circulating α 2-plasmin inhibitor-plasmin complexes (PAP), was diminished. Thus, the present study reflects the first instance of intervention in endotoxin mediated increase in circulating tPA and its activity in experimental endotoxemia in humans.

EXAMPLE 6

Protection From Endotoxin-Induced Hyperdynamic Circulatory State

[0071] Circulatory state assessments were performed as follows: Echocardiograms were performed using an Ultramark 9 echocardiography machine (Advanced Technology Laboratories (Bothell, WA)) with.a 2.25 MHz phased array probe that featured a steerable pulsed Doppler mode. All subjects underwent basal echocardiography studies at rest (basal) including M-mode measurements, 2-D imaging from parasternal, apical and subcostal views, color-coded Doppler imaging and pulsed Doppler measurements. Optimal parasternal and apical windows were obtained and marked on the subject's skin. All gain settings, sample volume size and depth and Doppler output settings were noted for each subject and carefully repeated at each measurement. The first basal study was performed several weeks before the first day of the endotoxin infusion. On each infusion day a basal study was performed in the early morning one hour prior to the infusion. The fourth basal study was obtained six to eight weeks after the last infusion. Averages of these four echocardiograms comprise the basal values. On the study days at timepoints 1:30, 2:30, 4:00, 5:00, 8:00, 12:00 hours after start of infusion the following measurements were performed: M-mode; Left Ventricular End Diastolic Diameter (LVEDD), Left Ventricular End I Systolic Diameter (LVESD). Interventricular Septal Thickness (IVS), Left Posterior Wall Thickness (LVPW), Dimension of the Aortic Root (Ao) and the Left Atrium (LA), tracings of the mitral valve motion and the aortic valve motion. 2-D: Standard parasternal long-axis and short-axis views, apical, and subcostal views were made to assess diameters, wall motion and aspect of mitral, aortic and tricuspid valve apparatus. Careful measurement of the diameter of the left ventricular outflow tract (D_{tvot}) at its narrowest point approximately one centimeter below the aortic valve was performed in the parasternal long axis view on an early systolic still frame after aortic valve opening. At every time point at least six measurements were made. Color-Doppler: Mitral valve flow and tricuspid valve flow and regurgitation were assessed if present. Pulsed Doppler measurement: At the basal studies and on the study days at time points 0:30, 1:00, 1:30, 2:00, 2:30, 3:00, 3:30, 4:00, 4:30, 5:00, 6:00, 8:00, 10:00, 12:00 hours after the start of infusion a pulsed Doppler measurement of the systolic flow in the center of the left ventricular outflow tract from the apical window was obtained. Ten consecutive Doppler flow tracings were recorded on videotape. Heart rate, temperature and blood pressure were simultaneously recorded.

[0072] Following completion of the study off-line, the Velocity Time Integral (VTI) or spectral area in meters given by the sum of $Vi*_{\delta t}$ were traced from the videotapes. Also the maximal velocity V_{max} was noted from the VTI tracings.

Averages were obtained from ten consecutive beats.

[0073] Equations employed to analyze results were as follows:

(1) Left Ventricular End Diastolic Volume (mL):

$$LVEDV = (7.0/(2.4+LVEDD))xLVEDD^3)/100;$$

(2) Left Ventricular End Systolic Volume (mL):

$$LVESV = (7.0/(2.4+LVESD))xLVESD^3)/100;$$

(3) Fractional Shortening (%):

$$FS = (LVEDD - LVESD)/LVEDD)x100;$$

(4) Ejection Fraction (%):

$$EF = (LVEDV - LVESV)/LVEDV)x100;$$

(5) Cross Sectional $Area_{tvot}$ (cm^2):

$$CSA = \pi (D_{tvot})^2/4;$$

(6) Stroke Volume (mL):

$$SV = CSA \times VTI \times 100;$$

(7) Cardiac Output (CO) (L):

$$CO = SV \times HR/1000$$

where HR is Heart Rate (beats per minute);

(8) Cardiac Index ($L/min/m^2$):

$$CI = CO/BSA$$

where BSA is Body Surface Area; and

(9) Body Surface Area (m^2):

$$BSA = (Height)^{0.725} \times (Weight)^{0.425} \times (0.007184)$$

(10) Systemic Vascular Resistance Index (dyne*sec/cm^5 per square meter):

$$SVRI = 80*(Mean\ Arterial\ Pressure - 6)/Cardiac\ Index.$$

[0074] Table 7, below, sets out the results of statistical analysis (as described in Example 1) of the primary left

ventricular function parameters, SVRI and CI. Graphic representations of SVRI and CI data are provided in Figures 18 and 19 respectively.

TABLE 7

PRIMARY LEFT VENTRICULAR FUNCTION			
Parameter	**Median % change in AUC[a]**	**p-value[b]**	**Statistical significance[c]**
SVRI	+28%	.0304[d]	S
CI	-13%	.0156	S

[a] AUC calculated from hours 0-6.

[b] p-value comparing $rBPI_{23}$ vs. placebo within each subject (Wilcoxon signed rank test).

[c] Statistical significance as determined by the Hochberg method (S=significant, NS=nonsignificant) applied to primary analysis parameters.

[d] Accounting for period effect.

[0075] Table 8, below, sets forth data concerning percent change in median result values and p-values for the collateral (secondary) assessments of other left ventricular function parameters.

TABLE 8

COLLATERAL LEFT VENTRICULAR FUNCTION		
Parameter	**Median % change in AUC[a]**	**p-value[b]**
AO	-4%	.55
CO	-11%	.0156
EF	-10%	.64
FS	-13%	.74
HR	-27%	.0078
IVS	+3%	.55
LA	-15%	.38
LVEDD	-4%	.95
LVEDV	-4%	.84
LVESD	+13%	.31[c]
LVESV	+14%	.31[c]
LVPW	+4%	.84
SV	+9%	.15
VTI	+1%	.55

[a] AUC calculated from hours 0-6.

[b] p-value comparing $rBPI_{23}$ vs. placebo within each subject (Wilcoxon signed rank test).

[c] Accounting for perlof effect.

[0076] Heart rate showed an increase in both study periods but less in the $rBPI_{23}$ treatment period. For the $rBPI_{23}$ treatment period, heart rate rose from 56 ± 2 beats/min at time 0 to a maximum of 77 ± 5 at 4:30 hours, dropping to 73 ± 3 at 6 hours, while for the placebo treatment period heart rate rose more rapidly from 61 ± 5 at time 0 to a maximum of 87 ± 4 at 3:30 hours and dropping to 75 ± 3 by 6 hours (27% reduction in AUC from time 0 to 6 hours, p=0.0078). The difference initial heart rate between the placebo and $rBPI_{23}$ period was mainly due to a difference in heart rate in one subject who started with heart rates of 87 and 74 at time 0 and 0:30 hours, respectively during the first infusion day (placebo), probably because of anxiety, and who had a heart rate of 55 at time 0 the second day.

[0077] Velocity time integrals (VT1) in the $rBPI_{23}$ treatment period showed an increase at 0:30 hours and 1 hour possibly related to the inital flush that six of the eight volunteers experienced. For the $rBPI_{23}$ treatment period VTI was 0.210 ± 0.009 m at time 0, 0.223 ± 0.008 m at 0:30 hours and 0.222 ± 0.006 m at 0:30 hours. For the placebo treatment period, VTI was 0.203 ± 0.008 m at time 0, 0.207 ± 0.006 m at 0:30 hours and 0.201 ± 0.007 m at 1 hour. From 5 hours onwards, there were consistently lower heart rates and higher VTI's in the $rBPI_{23}$ period.

[0078] V_{max} showed an increase in both infusion periods but the increase was attenuated in the $rBPI_{23}$ treatment period. (Median AUC 1.15 for $rBPI_{23}$, 2.29 for Placebo, p=0.061 by Wilcoxom Rank Sum test). V_{max} was examined statistically only in period 1 due to the presence of carryover effects between period 1 and 2. V_{max} rose from 1.05 ± 0.076 m/s at time 0 to 1.23 ± 0.08 m/s at 3:30 hours in the $rBPI_{23}$ treatment period and from 1.05 ± 0.05 m/s at time 0 to 1.29 ± 0.05 m/s at 3:30 hours in the placebo treatment period. The flow profile in the left ventricular outflow tract changes showing higher velocities but shorter ejection times resulting in conserved VTI.

[0079] Cross sectional area (CSA) was measured six times at each timepoint and averaged CSA did not change with heart rate and the average value of all measurements of the CSA of one infusion period for each subject was used as a constant in the equations. Average CSA in this group of eight volunteers was 4.37 ± 0.4 CM^2.

[0080] Cardiac index increased in both study periods but $rBPI_{23}$ infusion diminshed the endotoxin-induced rise in cardiac index significantly (Table 7: 13% reduction in AUC, p=0.0156). For the $rBPI_{23}$ treatment period, CI was 2.51 ± 0.16 (L/min/m^2) at time 0 and gradually rose to 3.27 ± 0.30 at 3:30 hours. For the placebo treatment period: CI was 2.64 ± 0.18 at time 0 rising to 3.98 ± 0.27 at 3:30 hours. At 4:30 hours, cardiac indices were elevated in both study periods compared to baseline and both gradually returned to baseline leves by 12 hours.

[0081] Systolic (SBP), Diastolic (DBP), and mean arterial blood pressure (MAP) decreased in both study periods and were not influenced by the $rBPI_{23}$ infusion.

[0082] Systemic vascular resistance index (SVRI) showed a decrease in both periods but the decrease was significantly less in the $rBPI_{23}$ infusion period (Table 7: 28% reduction in AUC, p=0.304). The SVRI was 2714.4 ± 204.0 at baseline falling to 1633.8 ± 88.5 at 3 hours in the placebo treatment period versus 2908.2 ± 205.2 at baseline and 2056.0 ± 145.2 at 3 hours in the $rBPI_{23}$ treatment period (Figure 18).

[0083] End diastolic volume and end systolic volume as measured by M-mode echocardiography were not different between treatment periods at any timepoint. Average end diastolic volume showed a gradual decline from 201 ± 13 mL at baseline to 188 ± 13 mL at 4 hours in the placebo period and 188 ± 11 mL at 4 hours in the $rBPI_{23}$ period. End systolic volume changed from 88 ± 6 mL at baseline to 74 ± 9 mL at 4 hours in the placebo period and 75 ± 7 mL at 4 hours in the $rBPI_{23}$ period.

[0084] M-mode ejection fraction showed a slight increase from 56 ± 0.9% at baseline to 60 ± 2.8% at 4 hours and 59 ± 1% at 12 hours in the $rBPI_{23}$ treatment period versus 62 ± 2.6% at 4 hours and 60 ± 2.8% at 12 hours in the placebo treatment period. Fractional shortening also showed an increase from 33.1 ± 0.7% at baseline to 36 ± 2.3% at 4 hours and 35 ± 0.9% at 12 hours in the $rBPI_{23}$ treatment period versus 38 ± 2.7% at 4 hours and 37 ± 2.2% at 12 hours in the placebo treatment period.

[0085] The above results establish that treatment of experimental endotoxemia in humans with a BPI protein product resulted in statistically significant modification of hyperdynamic changes in left ventricular function in response to endotoxin in circulation. The BPI protein product alleviated the decreases in systemic vascular resistance index and the concomitant increase in cardiac index which attend presence of endotoxin.

[0086] Numerous additional aspects and advantages of the present invention will be apparent to those skilled in the art upon consideration of the above illustrative examples of presently preferred practice thereof. For example, it will be apparent that human patients suffering from Gram negative bacteremia, accidental injection of endotoxin contaminated fluids or systemic release of endotoxin by translocation from the gut will benefit from administration of BPI protein products (by, *e.g.*, continuous intravenous infusion) to provide serologically and hematologically verifiable reduction in endotoxin mediated increases, for example, in levels of circulating cytokines and tissue plasminogen activator and changes in numbers of lymphocytes. It will also be apparent that BPI protein product administration will provide a beneficial adjunctive therapy for patients being treated with antibiotics and encountering entry of endotoxin into circulation as a result of bacterial lysis mediated by the antibiotic(s). Therefore only such limitations as appear in the appended claims should be placed upon the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: XOMA CORPORATION

(ii) TITLE OF INVENTION: Human Therapeutic Uses of BPI Protein Products

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 60606-6402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY INFORMATION:
(A) NAME: Michael F. Borun
(B) REGISTRATION NUMBER: 25,447
(C) REFERENCE/DOCKET NUMBER: 27129/32451

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312/474-6300
(B) TELEFAX: 312/474-0448
(C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 31..1491

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 124..1491

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(D) OTHER INFORMATION: "rBPI"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 10                  15                  20                  25

CAG AAG GAG CTG AAG AGG ATC AAG ATT CCT GAC TAC TCA GAC AGC TTT     246
Gln Lys Glu Leu Lys Arg Ile Lys Ile Pro Asp Tyr Ser Asp Ser Phe
                 30                  35                  40

AAG ATC AAG CAT CTT GGG AAG GGG CAT TAT AGC TTC TAC AGC ATG GAC     294
Lys Ile Lys His Leu Gly Lys Gly His Tyr Ser Phe Tyr Ser Met Asp
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ATC CGT GAA TTC CAG CTT CCC AGT TCC CAG ATA AGC ATG GTG CCC AAT     342
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GTG GGC CTT AAG TTC TCC ATC AGC AAC GCC AAT ATC AAG ATC AGC GGG     390
Val Gly Leu Lys Phe Ser Ile Ser Asn Ala Asn Ile Lys Ile Ser Gly
     75                  80                  85

AAA TGG AAG GCA CAA AAG AGA TTC TTA AAA ATG AGC GGC AAT TTT GAC     438
Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys Met Ser Gly Asn Phe Asp
 90                  95                  100                 105

CTG AGC ATA GAA GGC ATG TCC ATT TCG GCT GAT CTG AAG CTG GGC AGT     486
Leu Ser Ile Glu Gly Met Ser Ile Ser Ala Asp Leu Lys Leu Gly Ser
                110                 115                 120

AAC CCC ACG TCA GGC AAG CCC ACC ATC ACC TGC TCC AGC TGC AGC AGC     534
Asn Pro Thr Ser Gly Lys Pro Thr Ile Thr Cys Ser Ser Cys Ser Ser
            125                 130                 135

CAC ATC AAC AGT GTC CAC GTG CAC ATC TCA AAG AGC AAA GTC GGG TGG     582
His Ile Asn Ser Val His Val His Ile Ser Lys Ser Lys Val Gly Trp
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CTG ATC CAA CTC TTC CAC AAA AAA ATT GAG TCT GCG CTT CGA AAC AAG     630
Leu Ile Gln Leu Phe His Lys Lys Ile Glu Ser Ala Leu Arg Asn Lys
    155                 160                 165

ATG AAC AGC CAG GTC TGC GAG AAA GTG ACC AAT TCT GTA TCC TCC AAG     678
Met Asn Ser Gln Val Cys Glu Lys Val Thr Asn Ser Val Ser Ser Lys
170                 175                 180                 185

CTG CAA CCT TAT TTC CAG ACT CTG CCA GTA ATG ACC AAA ATA GAT TCT     726
Leu Gln Pro Tyr Phe Gln Thr Leu Pro Val Met Thr Lys Ile Asp Ser
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GAG ACC CTG GAT GTA CAG ATG AAG GGG GAG TTT TAC AGT GAG AAC CAC        822
Glu Thr Leu Asp Val Gln Met Lys Gly Glu Phe Tyr Ser Glu Asn His
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CAC AAT CCA CCT CCC TTT GCT CCA CCA GTG ATG GAG TTT CCC GCT GCC        870
His Asn Pro Pro Pro Phe Ala Pro Pro Val Met Glu Phe Pro Ala Ala
    235                 240                 245

CAT GAC CGC ATG GTA TAC CTG GGC CTC TCA GAC TAC TTC TTC AAC ACA        918
His Asp Arg Met Val Tyr Leu Gly Leu Ser Asp Tyr Phe Phe Asn Thr
250                 255                 260                 265

GCC GGG CTT GTA TAC CAA GAG GCT GGG GTC TTG AAG ATG ACC CTT ACA        966
Ala Gly Leu Val Tyr Gln Glu Ala Gly Val Leu Lys Met Thr Leu Arg
                270                 275                 280

GAT GAC ATG ATT CCA AAG GAG TCC AAA TTT CGA CTG ACA ACC AAG TTC       1014
Asp Asp Met Ile Pro Lys Glu Ser Lys Phe Arg Leu Thr Thr Lys Phe
            285                 290                 295

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Phe Gly Thr Phe Leu Pro Glu Val Ala Lys Lys Phe Pro Asn Met Lys
        300                 305                 310

ATA CAG ATC CAT GTC TCA GCC TCC ACC CCG CCA CAC CTG TCT GTG CAG       1110
Ile Gln Ile His Val Ser Ala Ser Thr Pro Pro His Leu Ser Val Gln
    315                 320                 325

CCC ACC GGC CTT ACC TTC TAC CCT GCC GTG GAT GTC CAG GCC TTT GCC       1158
Pro Thr Gly Leu Thr Phe Tyr Pro Ala Val Asp Val Gln Ala Phe Ala
330                 335                 340                 345

GTC CTC CCC AAC TCC TCC CTG GCT TCC CTC TTC CTG ATT GGC ATG CAC       1206
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                350                 355                 360

ACA ACT GGT TCC ATG GAG GTC AGC GCC GAG TCC AAC AGG CTT GTT GGA       1254
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GAG CTC AAG CTG GAT AGG CTG CTC CTG GAA CTG AAG CAC TCA AAT ATT       1302
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        380                 385                 390

GGC CCC TTC CCG GTT GAA TTG CTG CAG GAT ATC ATG AAC TAC ATT GTA       1350
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    395                 400                 405

CCC ATT CTT GTG CTG CCC AGG GTT AAC GAG AAA CTA CAG AAA GGC TTC       1398
Pro Ile Leu Val Leu Pro Arg Val Asn Glu Lys Leu Gln Lys Gly Phe
410                 415                 420                 425

CCT CTC CCG ACG CCG GCC AGA GTC CAG CTC TAC AAC GTA GTG CTT CAG       1446
Pro Leu Pro Thr Pro Ala Arg Val Gln Leu Tyr Asn Val Val Leu Gln
                430                 435                 440

CCT CAC CAG AAC TTC CTG CTG TTC GGT GCA GAC GTT GTC TAT AAA           1491
Pro His Gln Asn Phe Leu Leu Phe Gly Ala Asp Val Val Tyr Lys
            445                 450                 455

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ACCGGCTGCC TTTCCCCAGG GAATCCTCTC CAGATCTTAA CCAAGAGCCC CTTGCAAACT     1611
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TCTTCGACTC AGATTCAGAA ATGATCTAAA CACGAGGAAA CATTATTCAT TGGAAAAGTG    1671

CATGGTGTGT ATTTTAGGGA TTATGAGCTT CTTTCAAGGG CTAAGGCTGC AGAGATATTT    1731

CCTCCAGGAA TCGTGTTTCA ATTGTAACCA AGAAATTTCC ATTTGTGCTT CATGAAAAAA    1791

AACTTCTGGT TTTTTCATG TG                                               1813
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 487 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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-15                 -10                  -5                   1

Asn Pro Gly Val Val Val Arg Ile Ser Gln Lys Gly Leu Asp Tyr Ala
              5                  10                  15

Ser Gln Gln Gly Thr Ala Ala Leu Gln Lys Glu Leu Lys Arg Ile Lys
          20                  25                  30

Ile Pro Asp Tyr Ser Asp Ser Phe Lys Ile Lys His Leu Gly Lys Gly
      35                  40                  45

His Tyr Ser Phe Tyr Ser Met Asp Ile Arg Glu Phe Gln Leu Pro Ser
  50                  55                  60                  65

Ser Gln Ile Ser Met Val Pro Asn Val Gly Leu Lys Phe Ser Ile Ser
                  70                  75                  80

Asn Ala Asn Ile Lys Ile Ser Gly Lys Trp Lys Ala Gln Lys Arg Phe
              85                  90                  95

Leu Lys Met Ser Gly Asn Phe Asp Leu Ser Ile Glu Gly Met Ser Ile
          100                 105                 110

Ser Ala Asp Leu Lys Leu Gly Ser Asn Pro Thr Ser Gly Lys Pro Thr
      115                 120                 125

Ile Thr Cys Ser Ser Cys Ser Ser His Ile Asn Ser Val His Val His
  130                 135                 140                 145

Ile Ser Lys Ser Lys Val Gly Trp Leu Ile Gln Leu Phe His Lys Lys
                  150                 155                 160

Ile Glu Ser Ala Leu Arg Asn Lys Met Asn Ser Gln Val Cys Glu Lys
              165                 170                 175

Val Thr Asn Ser Val Ser Ser Lys Leu Gln Pro Tyr Phe Gln Thr Leu
          180                 185                 190

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Val Ala Pro Pro Ala Thr Thr Ala Glu Thr Leu Asp Val Gln Met Lys
  210                 215                 220                 225
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Gly Glu Phe Tyr Ser Glu Asn His His Asn Pro Pro Pro Phe Ala Pro
                230                 235                 240

Pro Val Met Glu Phe Pro Ala Ala His Asp Arg Met Val Tyr Leu Gly
            245                 250                 255

Leu Ser Asp Tyr Phe Phe Asn Thr Ala Gly Leu Val Tyr Gln Glu Ala
        260                 265                 270

Gly Val Leu Lys Met Thr Leu Arg Asp Asp Met Ile Pro Lys Glu Ser
    275                 280                 285

Lys Phe Arg Leu Thr Thr Lys Phe Phe Gly Thr Phe Leu Pro Glu Val
290                 295                 300                 305

Ala Lys Lys Phe Pro Asn Met Lys Ile Gln Ile His Val Ser Ala Ser
                310                 315                 320

Thr Pro Pro His Leu Ser Val Gln Pro Thr Gly Leu Thr Phe Tyr Pro
            325                 330                 335

Ala Val Asp Val Gln Ala Phe Ala Val Leu Pro Asn Ser Ser Leu Ala
        340                 345                 350

Ser Leu Phe Leu Ile Gly Met His Thr Thr Gly Ser Met Glu Val Ser
    355                 360                 365

Ala Glu Ser Asn Arg Leu Val Gly Glu Leu Lys Leu Asp Arg Leu Leu
370                 375                 380                 385

Leu Glu Leu Lys His Ser Asn Ile Gly Pro Phe Pro Val Glu Leu Leu
                390                 395                 400

Gln Asp Ile Met Asn Tyr Ile Val Pro Ile Leu Val Leu Pro Arg Val
            405                 410                 415

Asn Glu Lys Leu Gln Lys Gly Phe Pro Leu Pro Thr Pro Ala Arg Val
        420                 425                 430

Gln Leu Tyr Asn Val Val Leu Gln Pro His Gln Asn Phe Leu Leu Phe
    435                 440                 445

Gly Ala Asp Val Val Tyr Lys
450                 455
```

Claims

1. A method for treatment of humans exposed to bacterial endotoxin in circulation comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating tumor necrosis factor

and interleukin 6.

2. A method for treatment of humans exposed to bacterial endotoxin in circulation comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating interleukin 8 and in neutrophil degranulation as **characterized by** increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes.

3. A method for treatment of humans exposed to bacterial endotoxin in circulation comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated changes in numbers of circulating lymphocytes.

4. A method for treatment of humans exposed to bacterial endotoxin in circulation comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating tissue plasminogen activator and tissue plasminogen activator activity.

5. A method for treatment of humans exposed to bacterial endotoxin in circulation comprising administering a BPI protein product in an amount effective to alleviate endotoxin-mediated decreases in systemic vascular resistance index.

6. In a human therapeutic method involving administration of a bacterial antibiotic, the improvement comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating tumor necrosis factor and interleukin 6.

7. In a human therapeutic method involving administration of a bacterial antibiotic, the improvement comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating interleukin 8 and in neutrophil degranulation as **characterized by** increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes.

8. In a human therapeutic method involving administration of a bacterial antibiotic, the improvement comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated changes in numbers of circulating lymphocytes.

9. In a human therapeutic method involving administration of a bacterial antibiotic, the improvement comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated increase in circulating tissue plasminogen activator and tissue plasminogen activator activity.

10. In a human therapeutic method involving administration of a bacterial antibiotic, the improvement comprising administering a BPI protein product in an amount effective to alleviate endotoxin mediated decreases in systemic vascular resistance index.

11. Use of a BPI protein product in the manufacture of a medicament for alleviating endotoxin mediated increases in circulating tumor necrosis factor and interleukin 6 in humans exposed to bacterial endotoxin in circulation.

12. Use of a BPI protein product in the manufacture of a medicament for alleviating endotoxin mediated increases in circulating interleukin 8 and in neutrophil degranulation as **characterized by** increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes in humans exposed to bacterial endotoxin in circulation.

13. Use of a BPI protein product in the manufacture of a medicament for alleviating endotoxin mediated changes in numbers of circulating lymphocytes in humans exposed to bacterial endotoxin in circulation.

14. Use of a BPI protein product in the manufacture of a medicament for alleviating endotoxin mediated increases in circulating tissue plasminogen activator and tissue plasminogen activator activity in humans exposed to bacterial endotoxin in circulation.

15. Use of a BPI protein product in the manufacture of a medicament for alleviating endotoxin mediated decreases in systemic vascular resistance index in humans exposed to bacterial endotoxin in circulation.

16. Use of a BPI protein product in combination with a bacterial antibiotic for the manufacture of a medicament for

alleviating endotoxin mediated increases in circulating tumor necrosis factor and interleukin 6 in humans exposed to bacterial endotoxin in circulation.

17. Use of a BPI protein product in combination with a bacterial antibiotic for the manufacture of a medicament for alleviating endotoxin mediated increases in circulating interleukin 8 and in neutrophil degranulation as **characterized by** increased circulating lactoferrin and/or elastase/α 1 antitrypsin complexes in humans exposed to bacterial endotoxin in circulation.

18. Use of a BPI protein product in combination with a bacterial antibiotic for the manufacture of a medicament for alleviating endotoxin mediated changes in numbers of circulating lymphocytes in humans exposed to bacterial endotoxin in circulation.

19. Use of a BPI protein product in combination with a bacterial antibiotic for the manufacture of a medicament for alleviating endotoxin mediated increases in circulating tissue plasminogen activator and tissue plasminogen activator activity in humans exposed to bacterial endotoxin in circulation.

20. Use of a BPI protein product in combination with a bacterial antibiotic for the manufacture of a medicament for alleviating endotoxin mediated decreases in systemic vascular resistance index in humans exposed to bacterial endotoxin in circulation.

FIG. 1

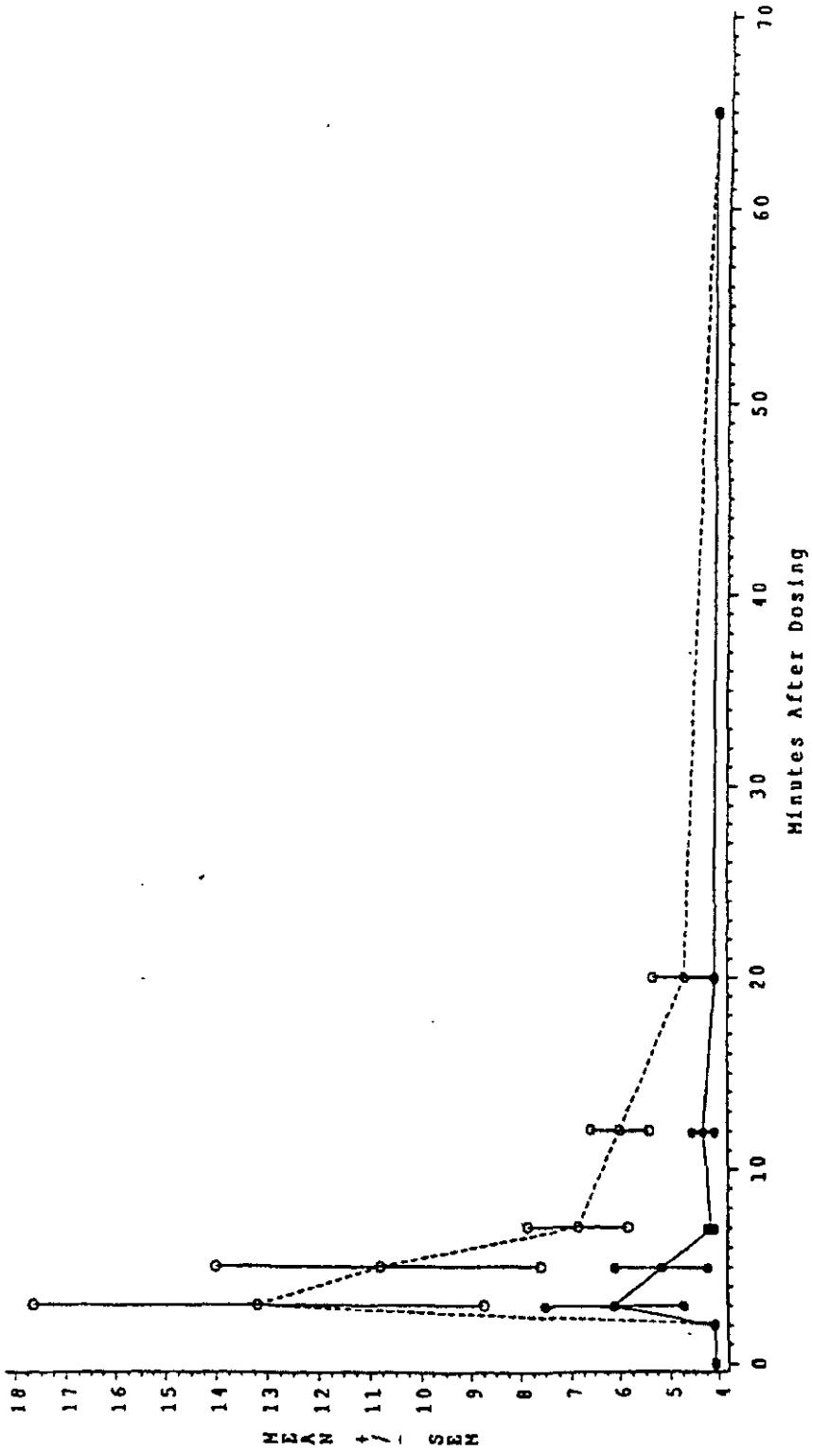

MEAN +/- SEM
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
0
10
20
30
40
50
60
70
Minutes After Dosing

FIG. 2

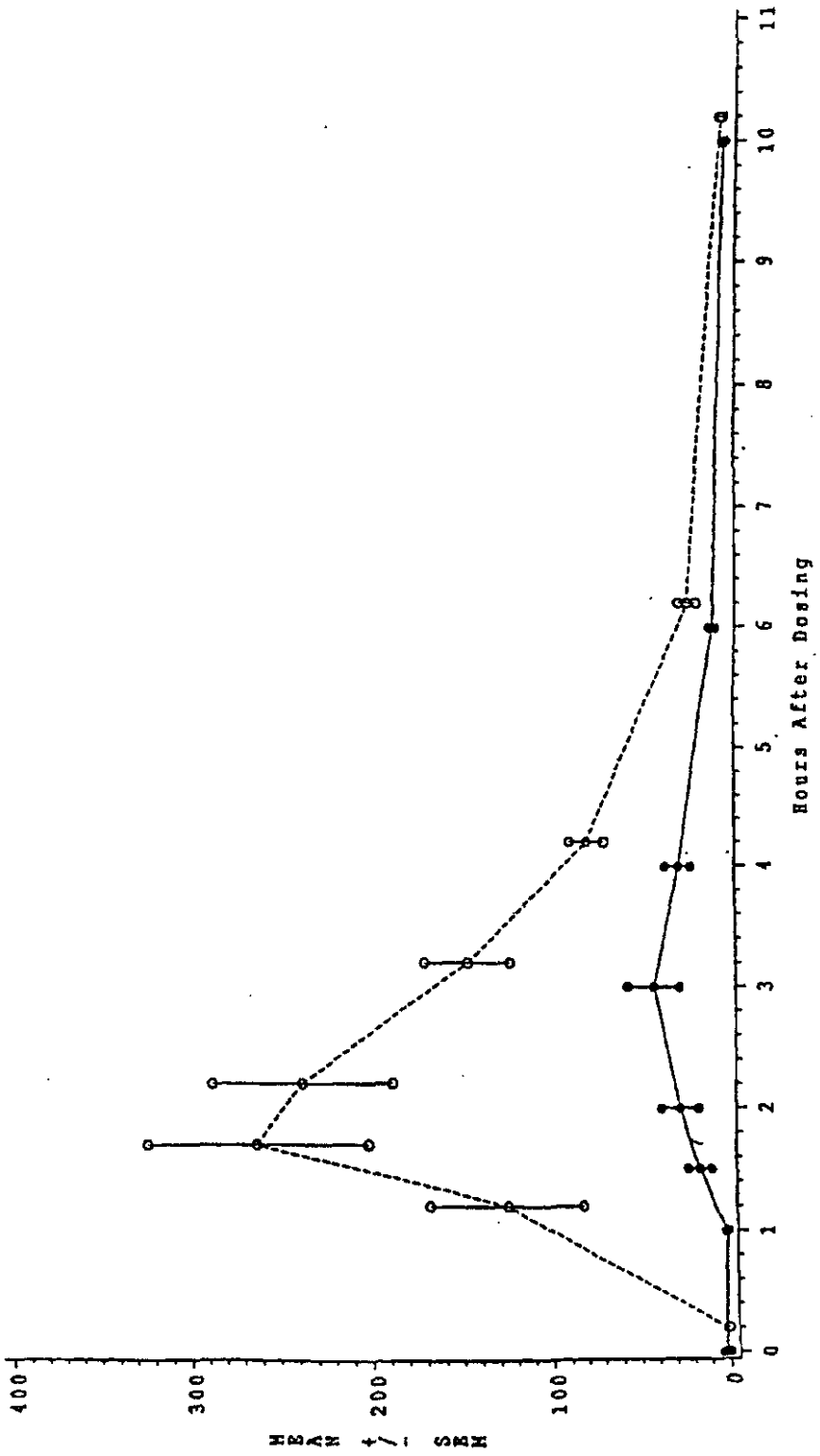

MEAN +/- SEM
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
Hours After Dosing

FIG. 3

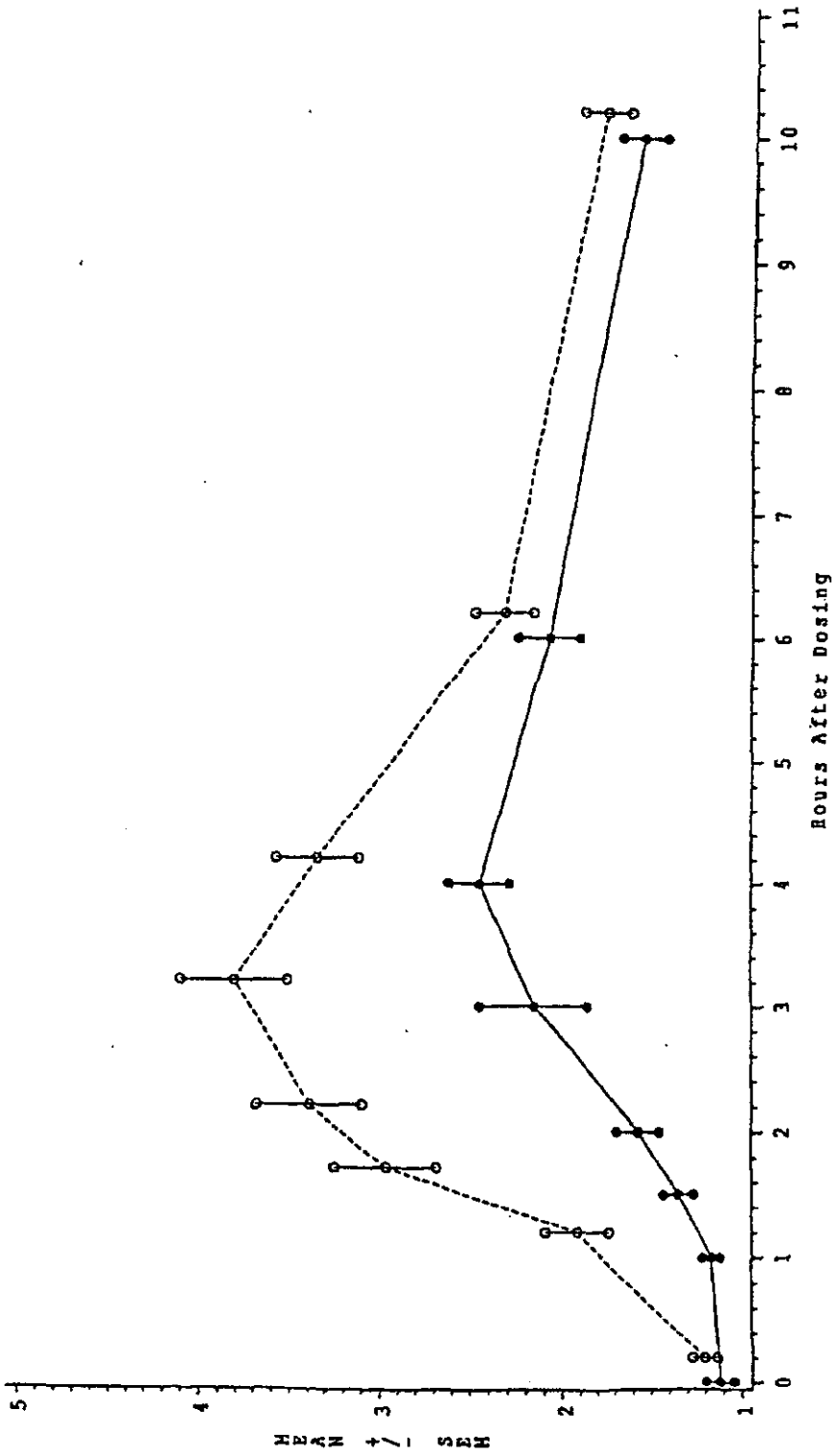


FIG. 4

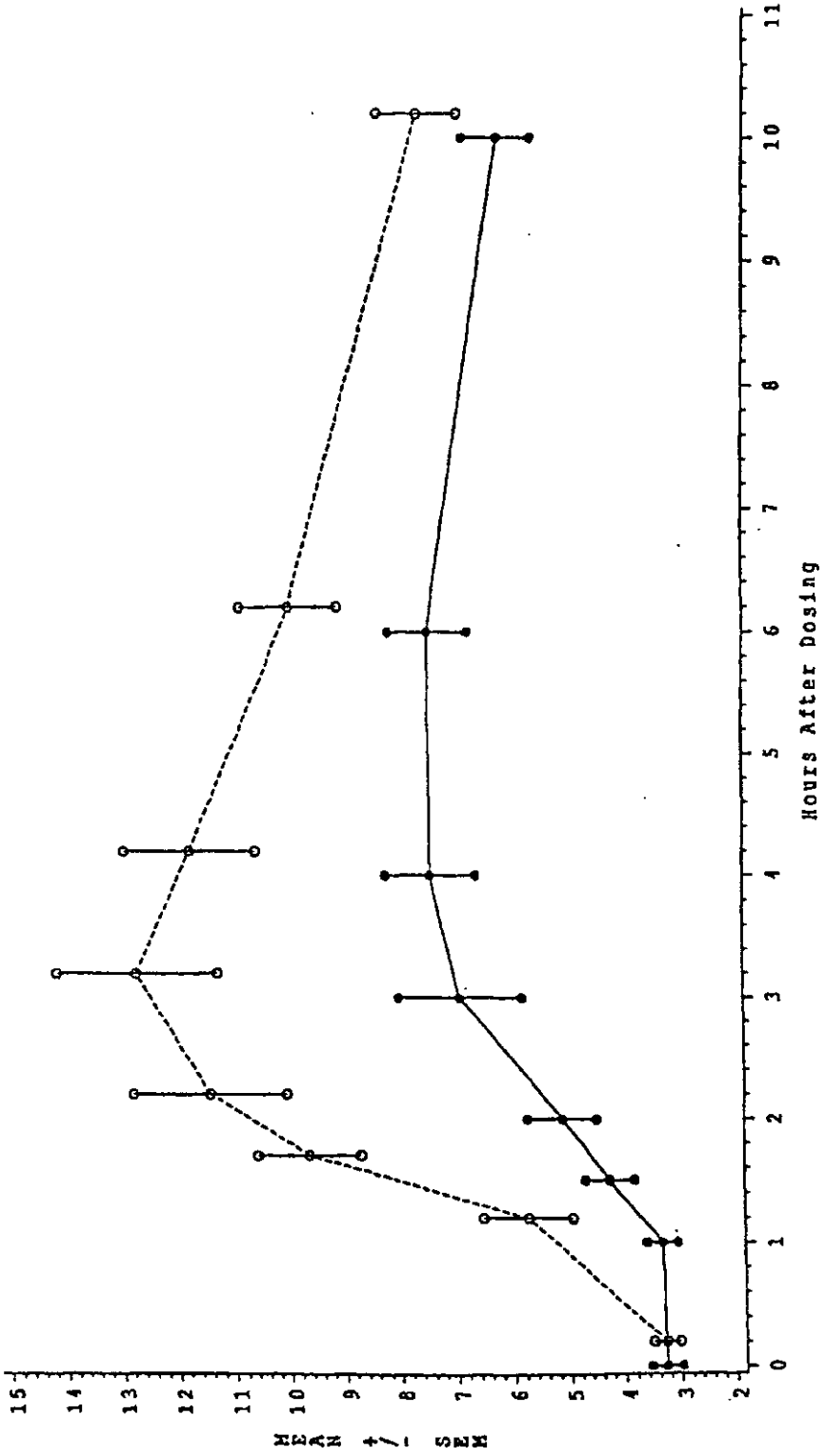

MEAN +/- SEM
15
14
13
12
11
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
6
7
8
9
10
11
Hours After Dosing

FIG. 5

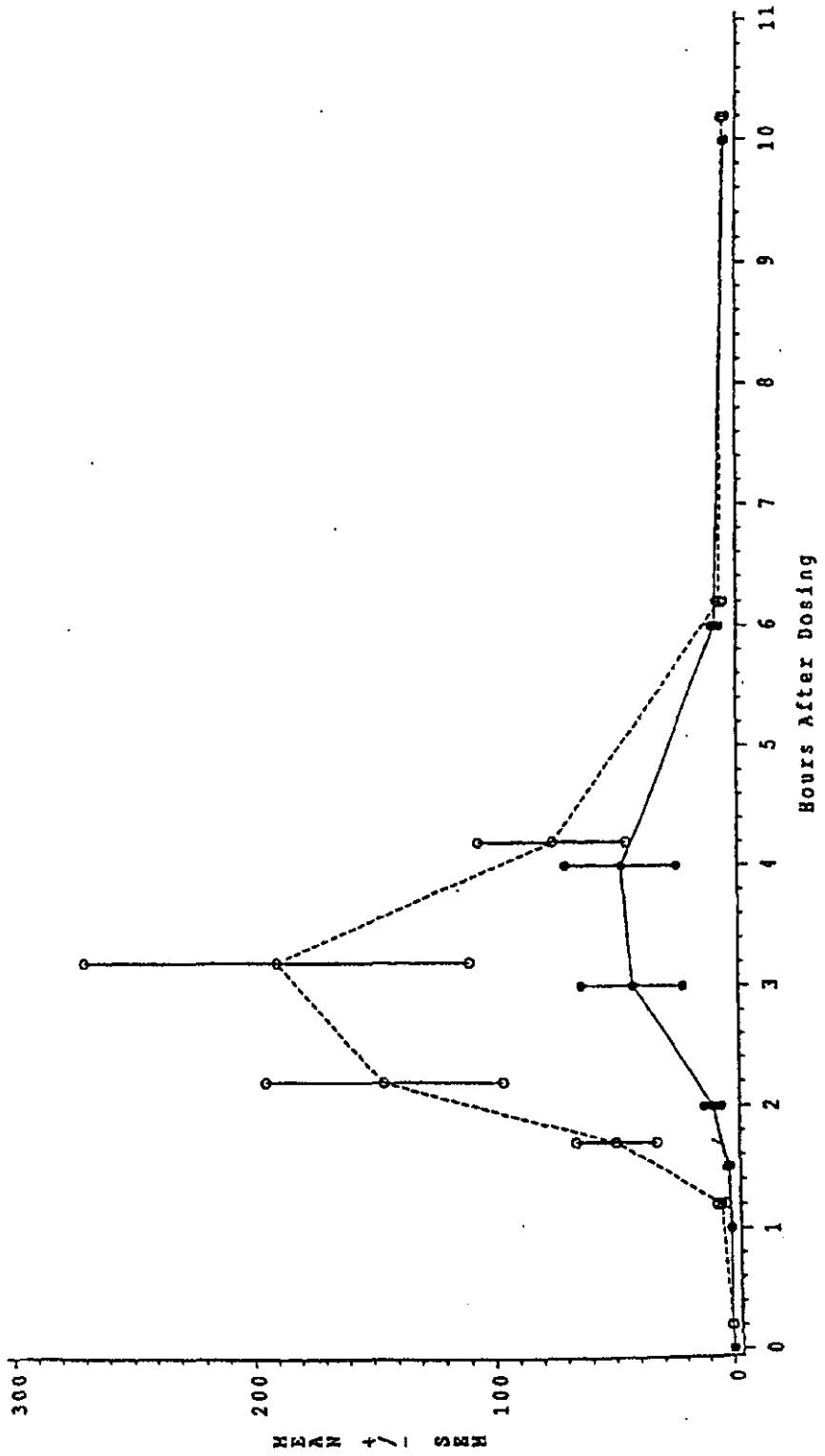


FIG. 6

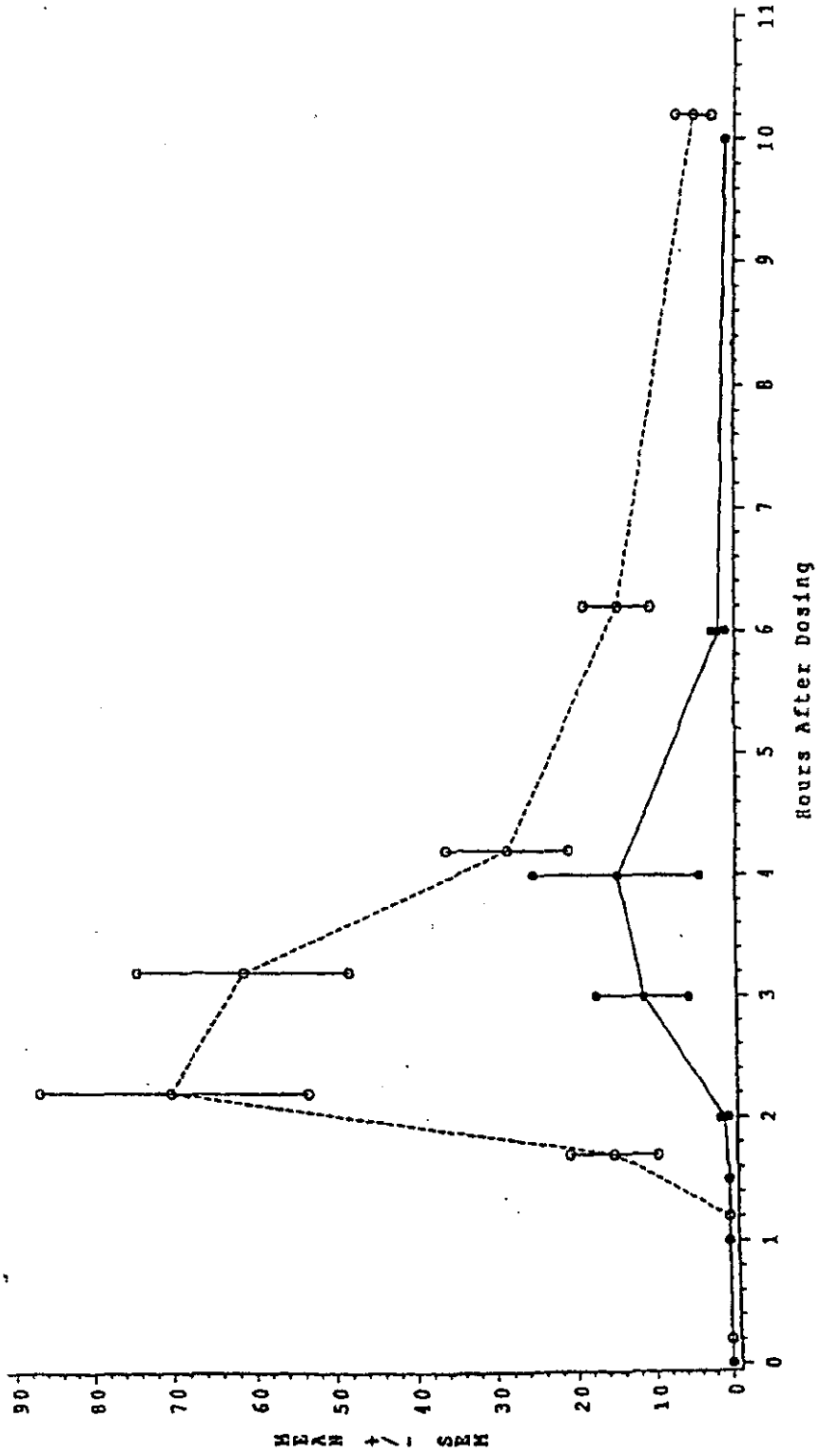

MEAN +/- SEM
0
10
20
30
40
50
60
70
80
90
Hours After Dosing
0
1
2
3
4
5
6
7
8
9
10
11

FIG. 7

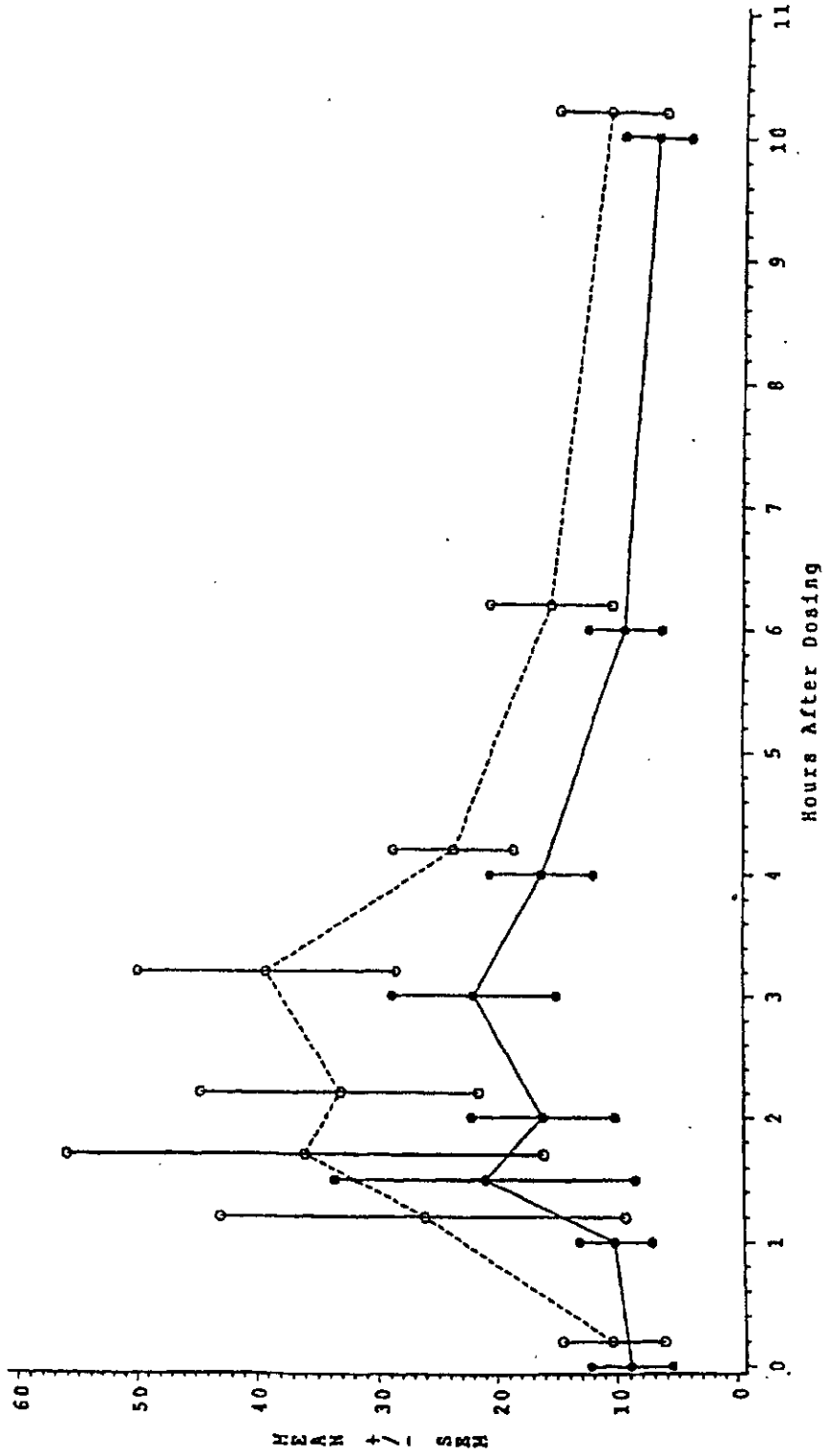

Hours After Dosing
0
1
2
3
4
5
6
7
8
9
10
11
MWS +/- MWSM
0
10
20
30
40
50
60

FIG. 8

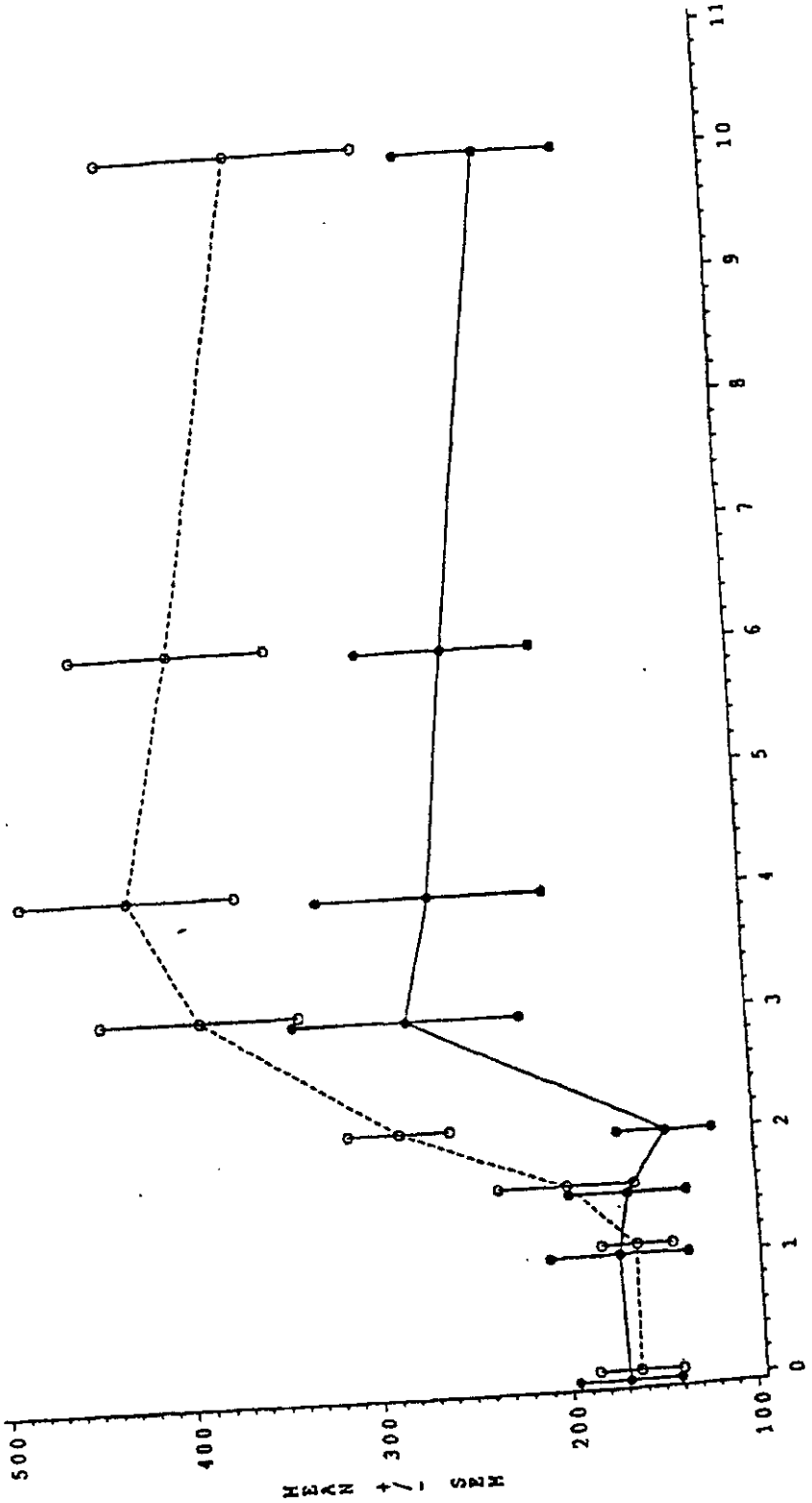


FIG. 9

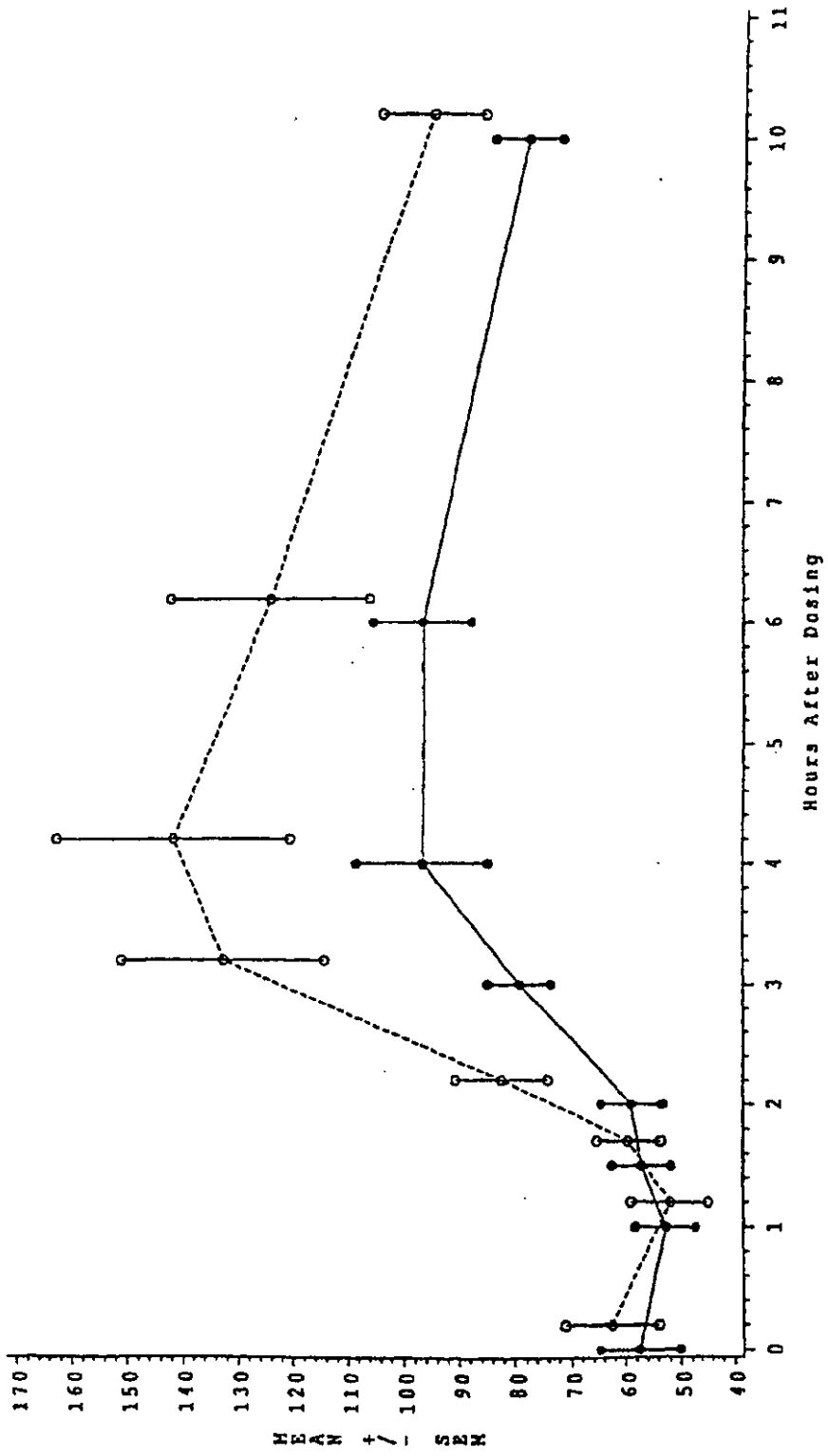

MEAN +/- SEM
170
160
150
140
130
120
110
100
90
80
70
60
50
40
0
1
2
3
4
5
6
7
8
9
10
11
Hours After Dosing

FIG. 10

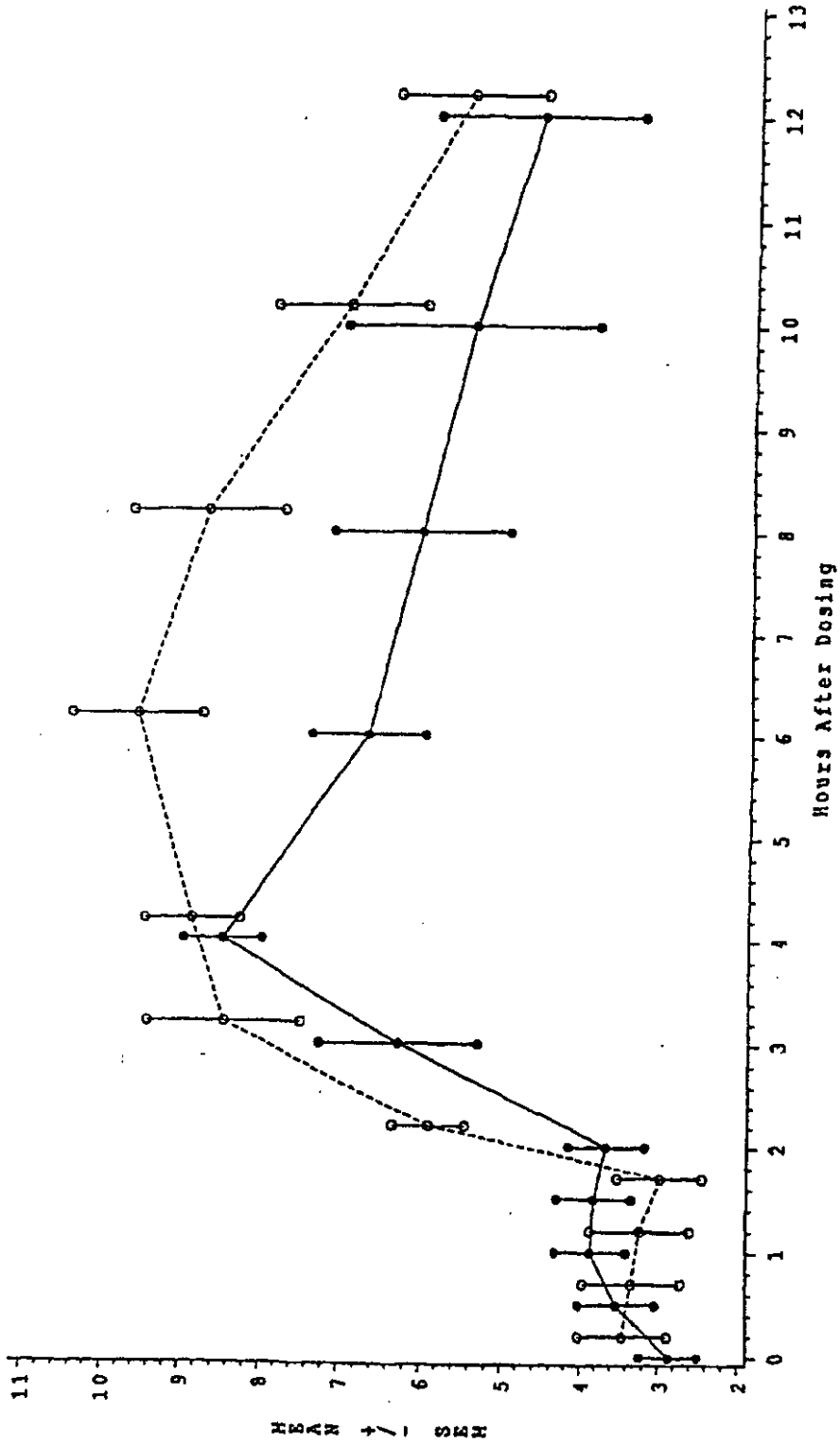

MEAN +/- SEM
Hours After Dosing
0
1
2
3
4
5
6
7
8
9
10
11
12
13
2
3
4
5
6
7
8
9
10
11

FIG. 11

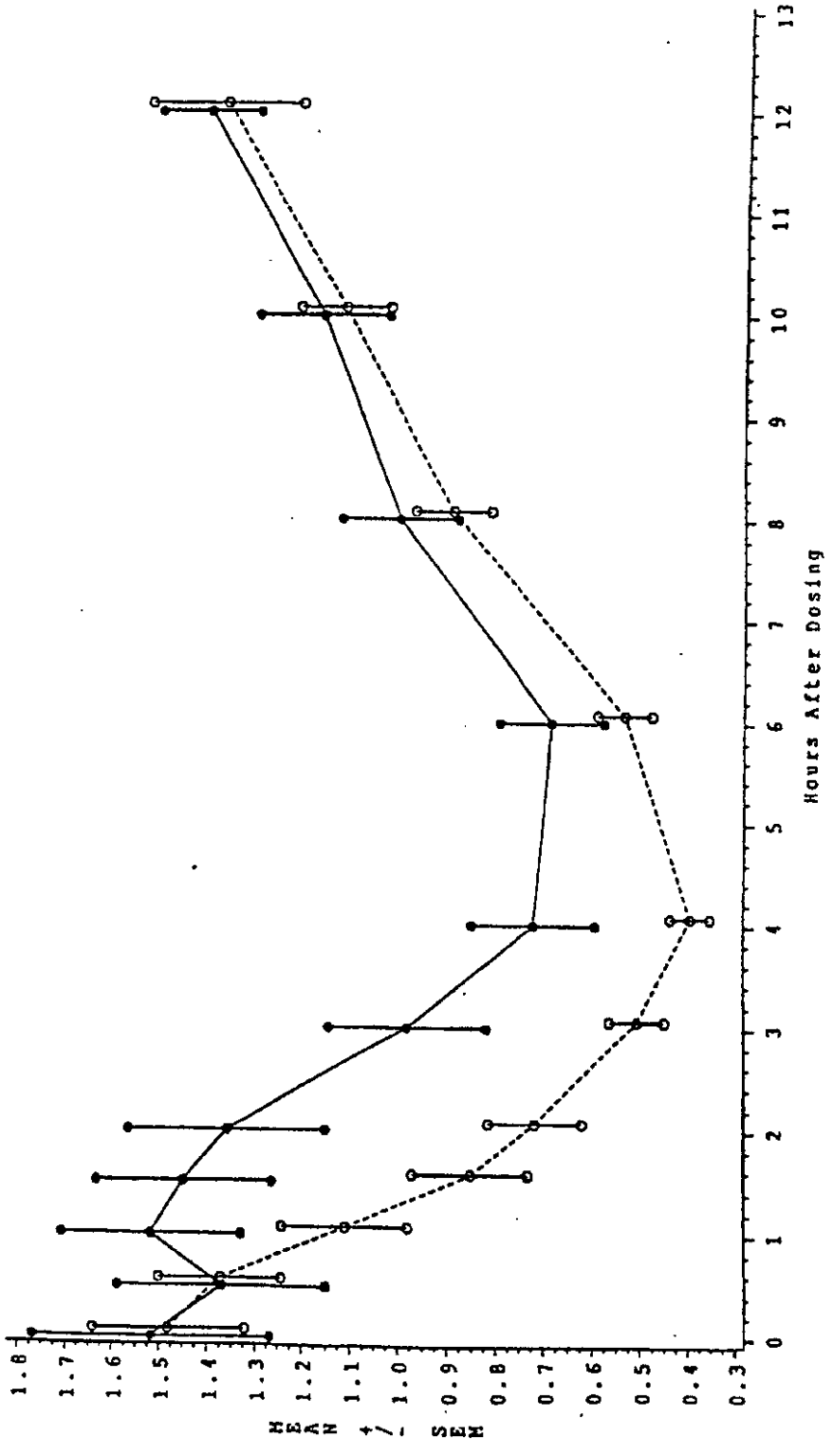

MEAN +/- SEM
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Hours After Dosing

FIG. 12

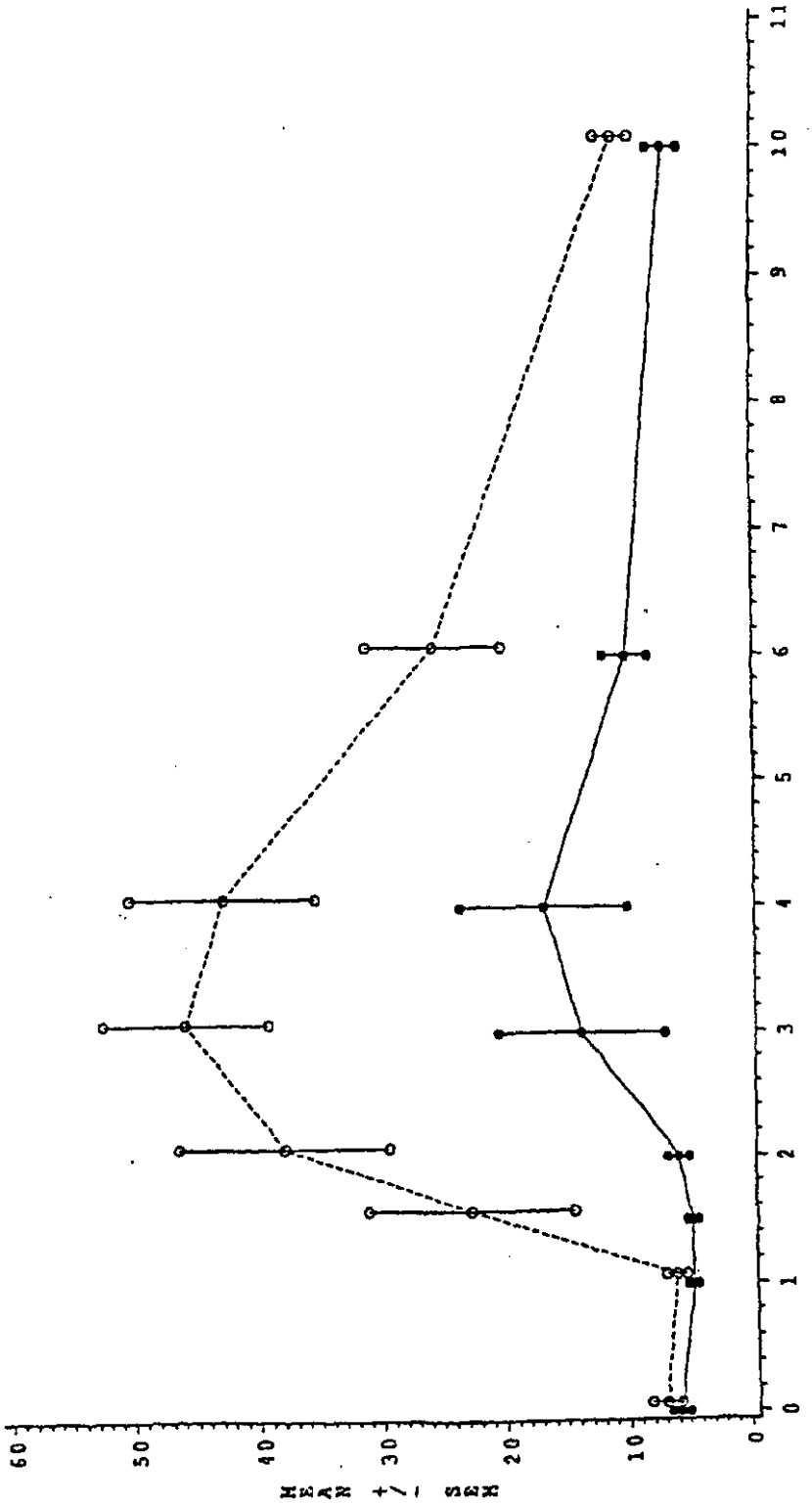


FIG. 13

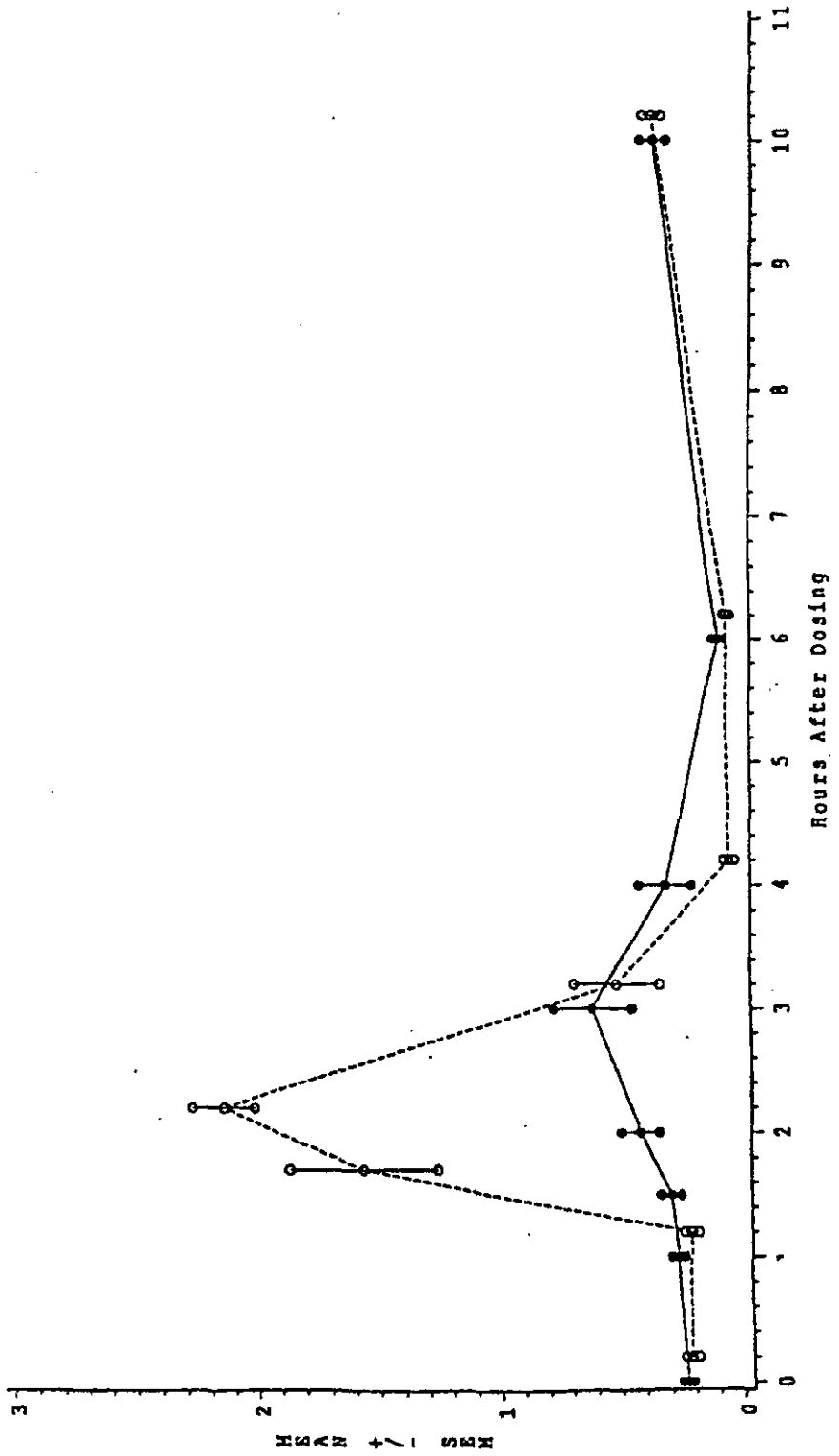


FIG. 14

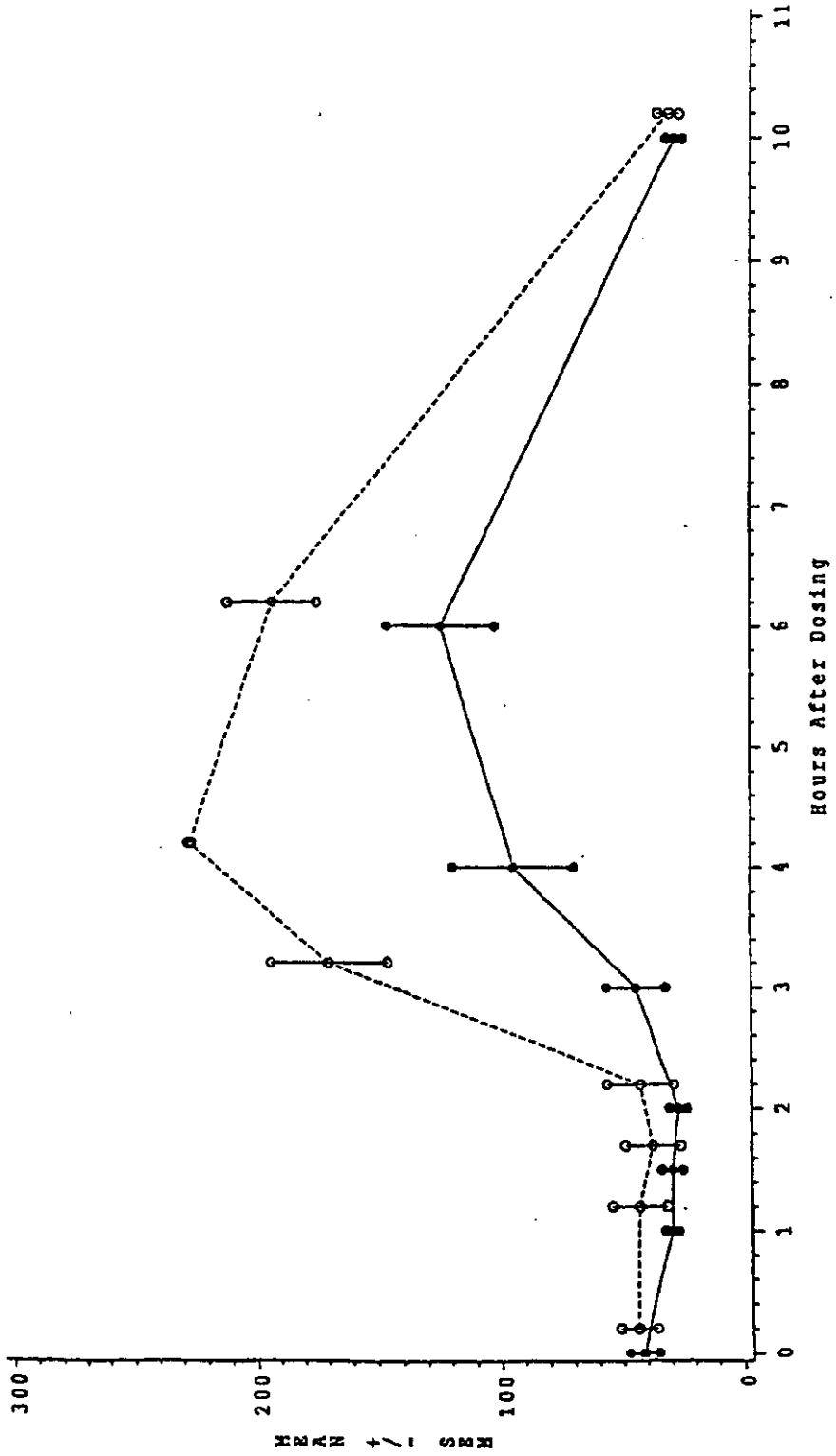


FIG. 15

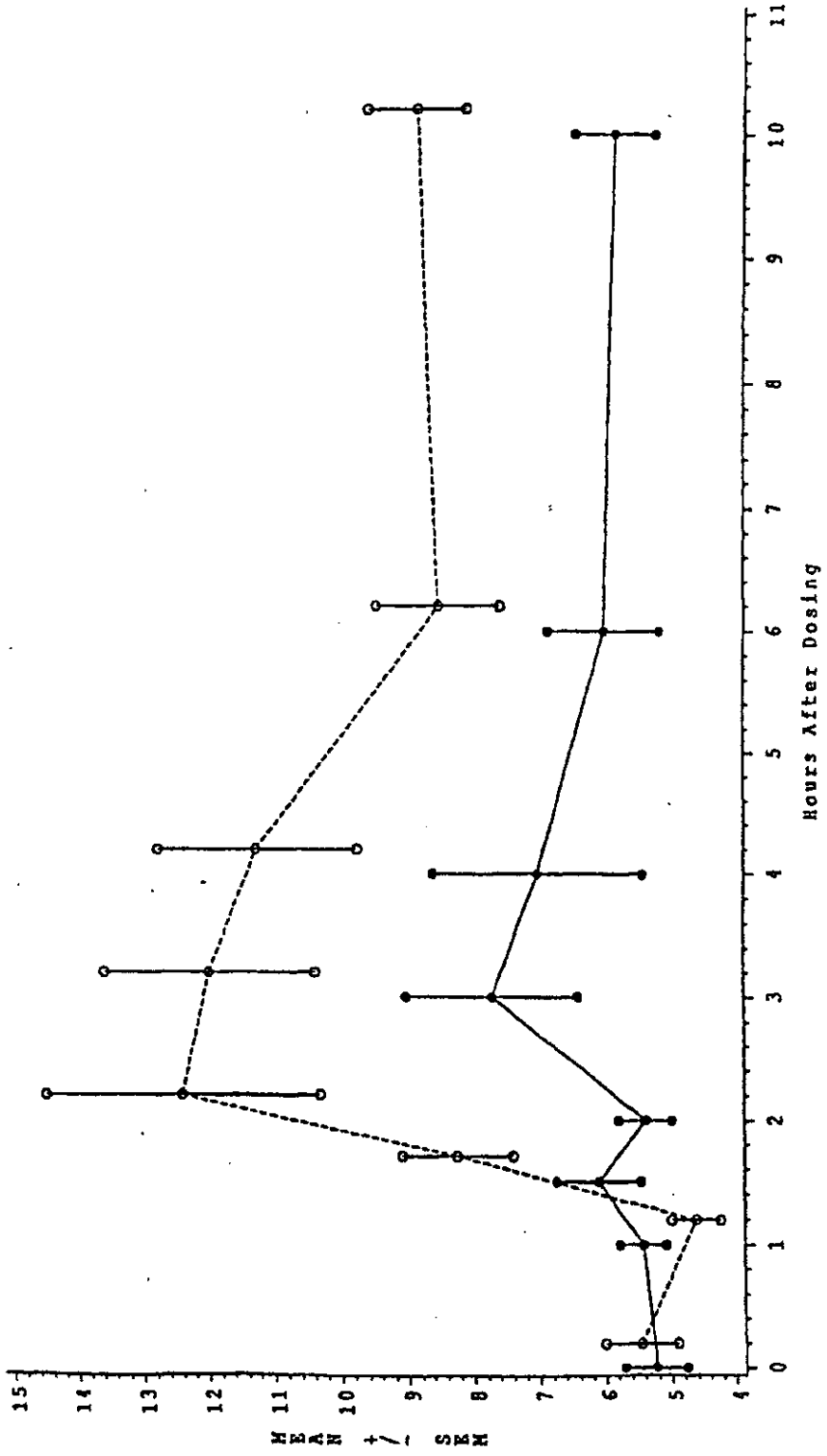

MEAN +/- SEM
15
14
13
12
11
10
9
8
7
6
5
4
0
1
2
3
4
5
6
7
8
9
10
11
Hours After Dosing

FIG. 16

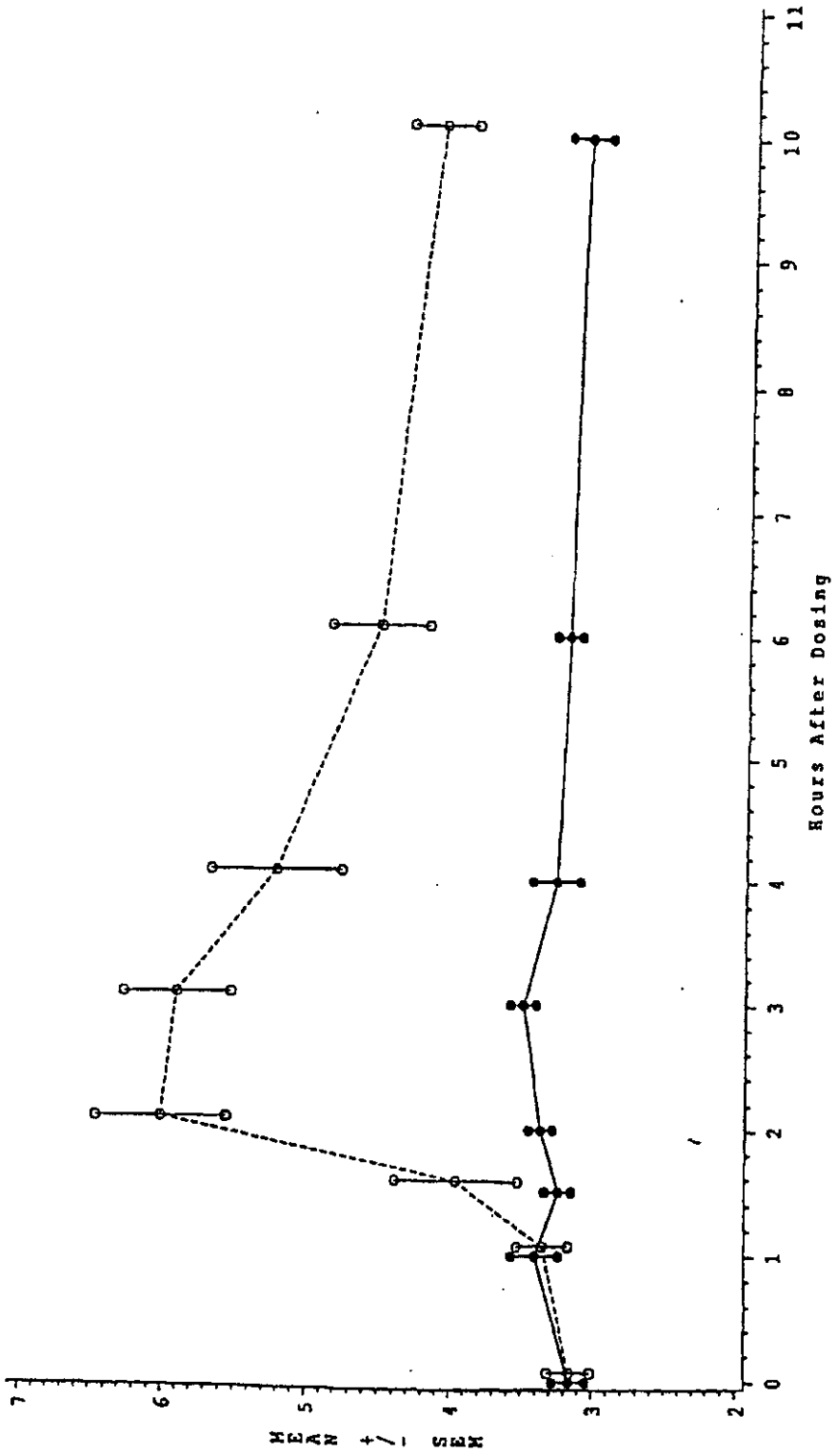


FIG. 17

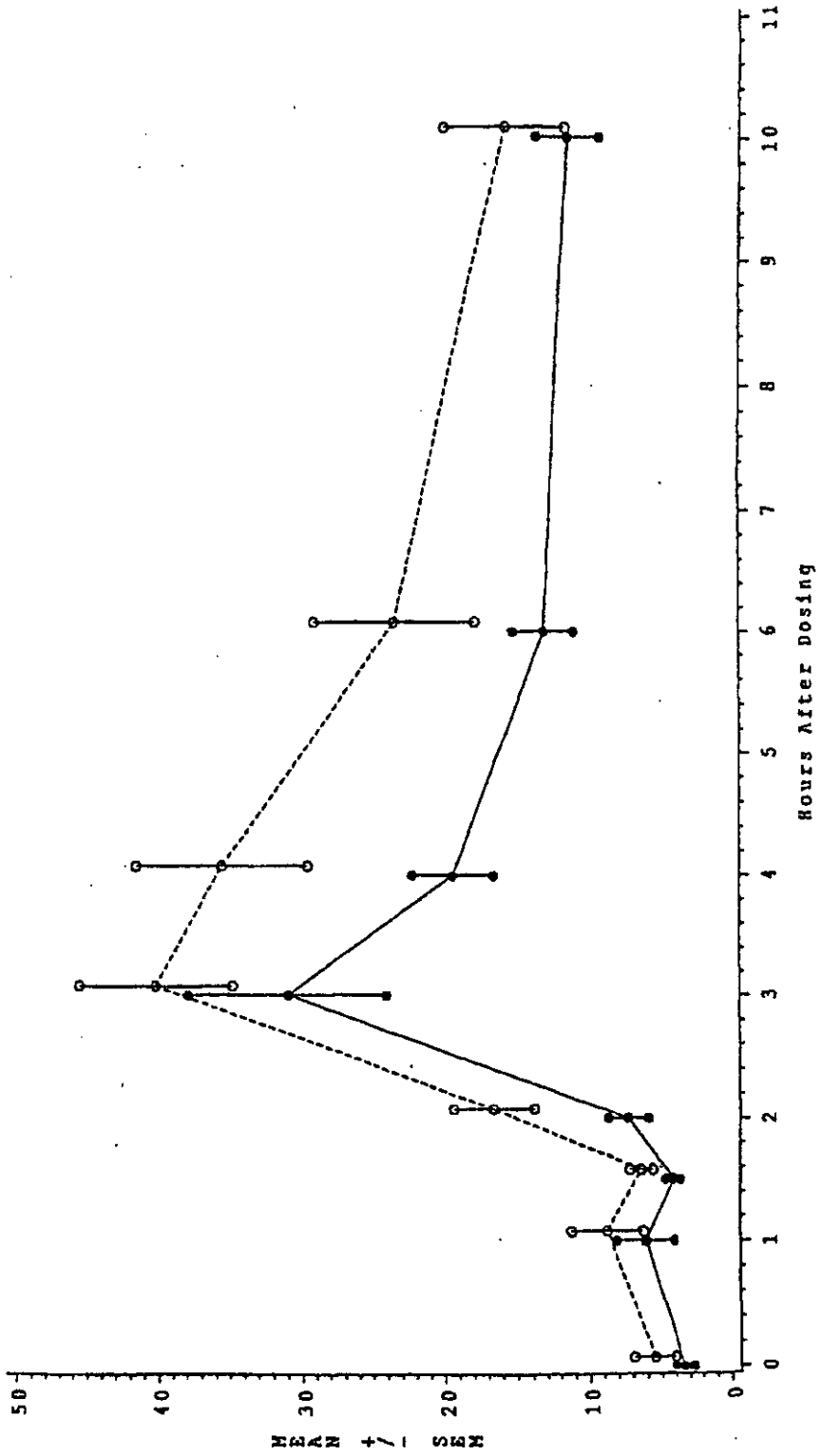


FIG. 18

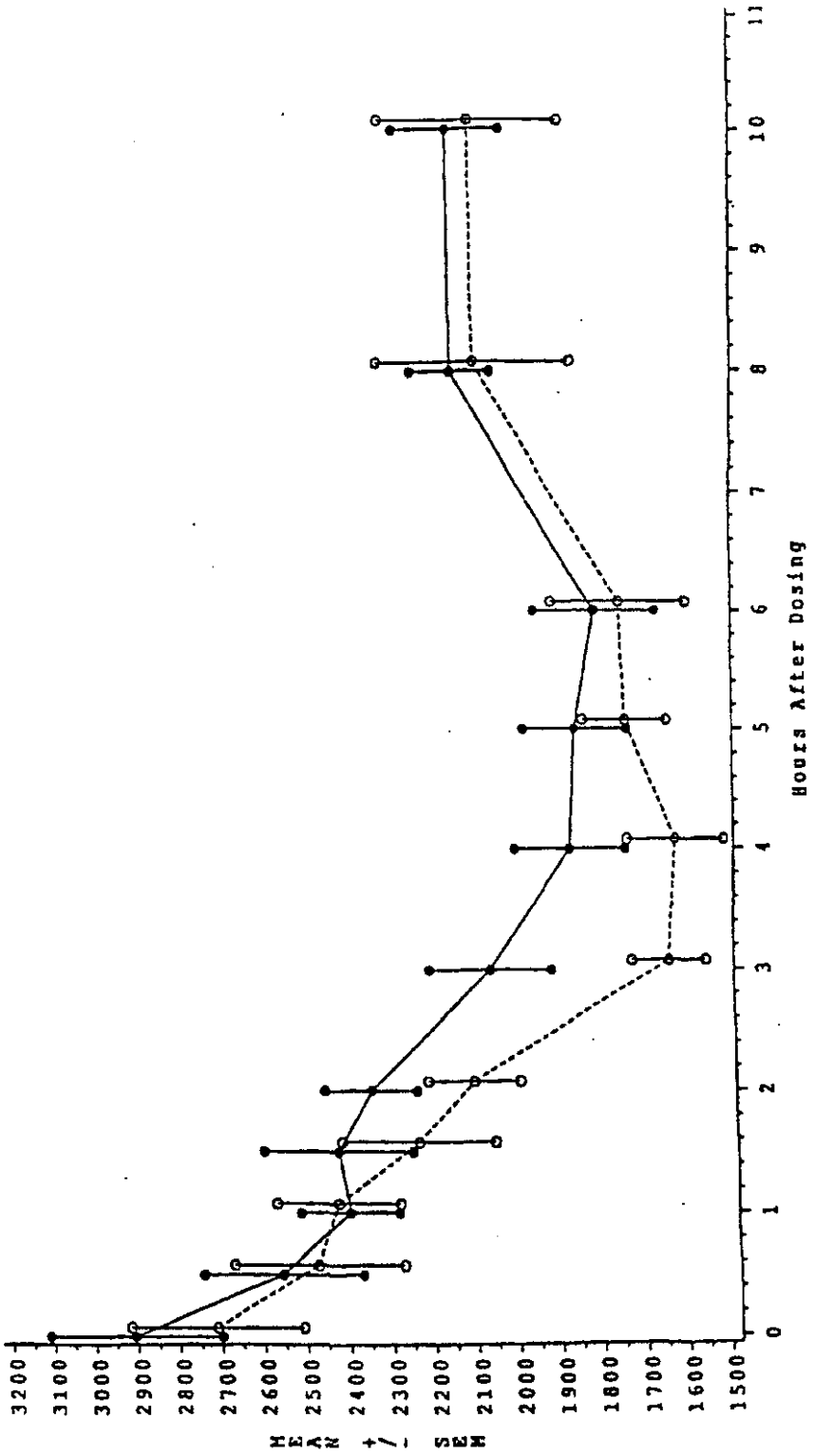
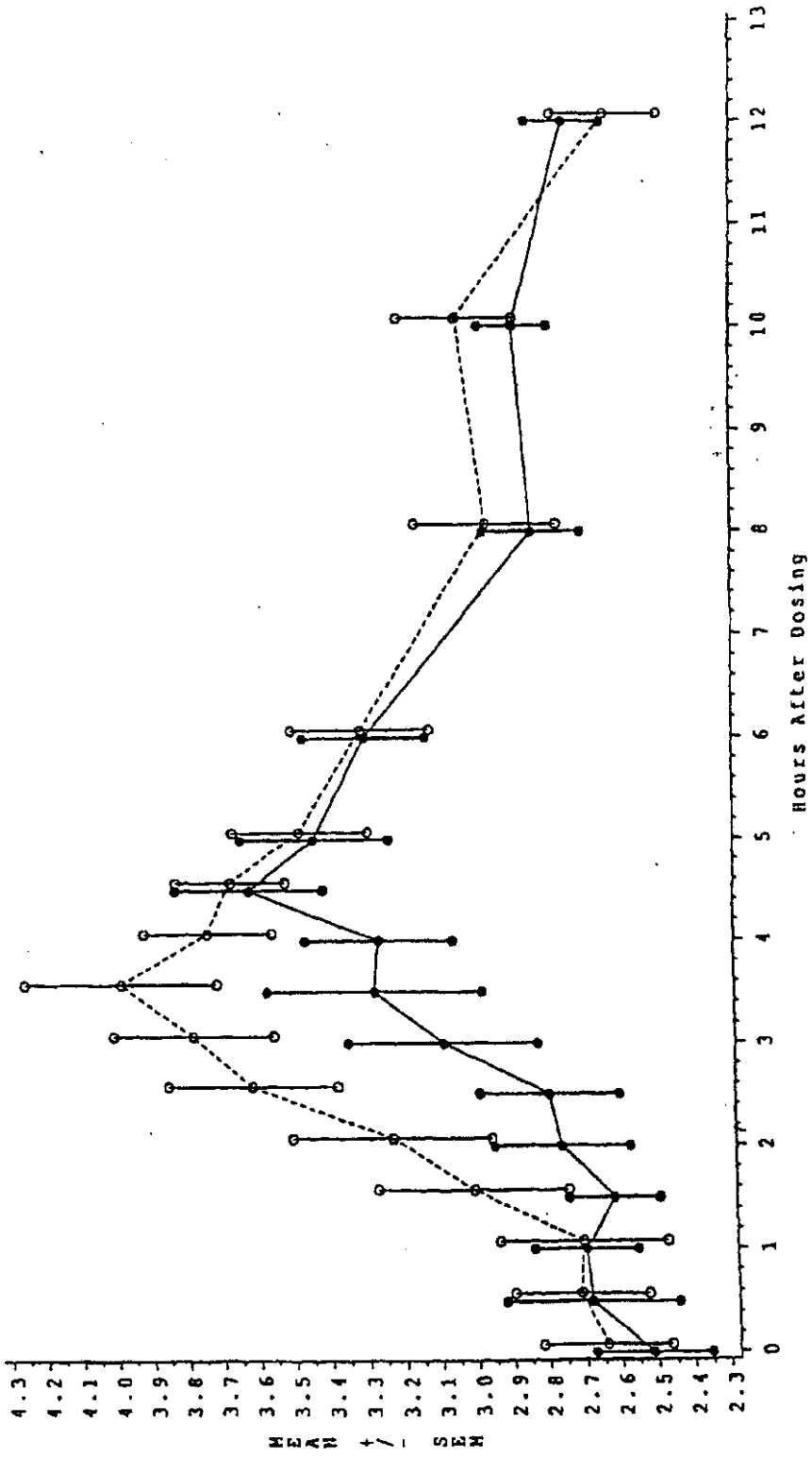

MEAN +/- SEM
3200
3100
3000
2900
2800
2700
2600
2500
2400
2300
2200
2100
2000
1900
1800
1700
1600
1500
0
1
2
3
4
5
6
7
8
9
10
11
Hours After Dosing

FIG. 19


MEAN +/- SEM
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
2.7
2.6
2.5
2.4
2.3
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Hours After Dosing

TJH:27323

(54) BPI 蛋白質產品的人類醫療用途

(57) 公開治療在循環被暴露於細菌性內毒素的人類的方法，利用施以殺菌/滲透度增加(BPI) 蛋白質產品，在血清和血液上能被證實減少由內毒素引起循環細胞激素的增加，在這治療中觀察淋巴球數裡纖溶作用和凝固作用的因素和變化，同時觀察減輕由內毒素引起的系統血管阻力指數(SVRI)和心博輸出量指數(CI)的同步增加。