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(54) Titre : UTILISTION D'AGENTS PROTECTEURS CONTRE DES ESPECES REAGISSANT A L'OXYGENE  
 (54) Title: USE OF PROTECTIVE AGENTS AGAINST REACTIVE OXYGEN SPECIES

(57) **Abrégé/Abstract:**

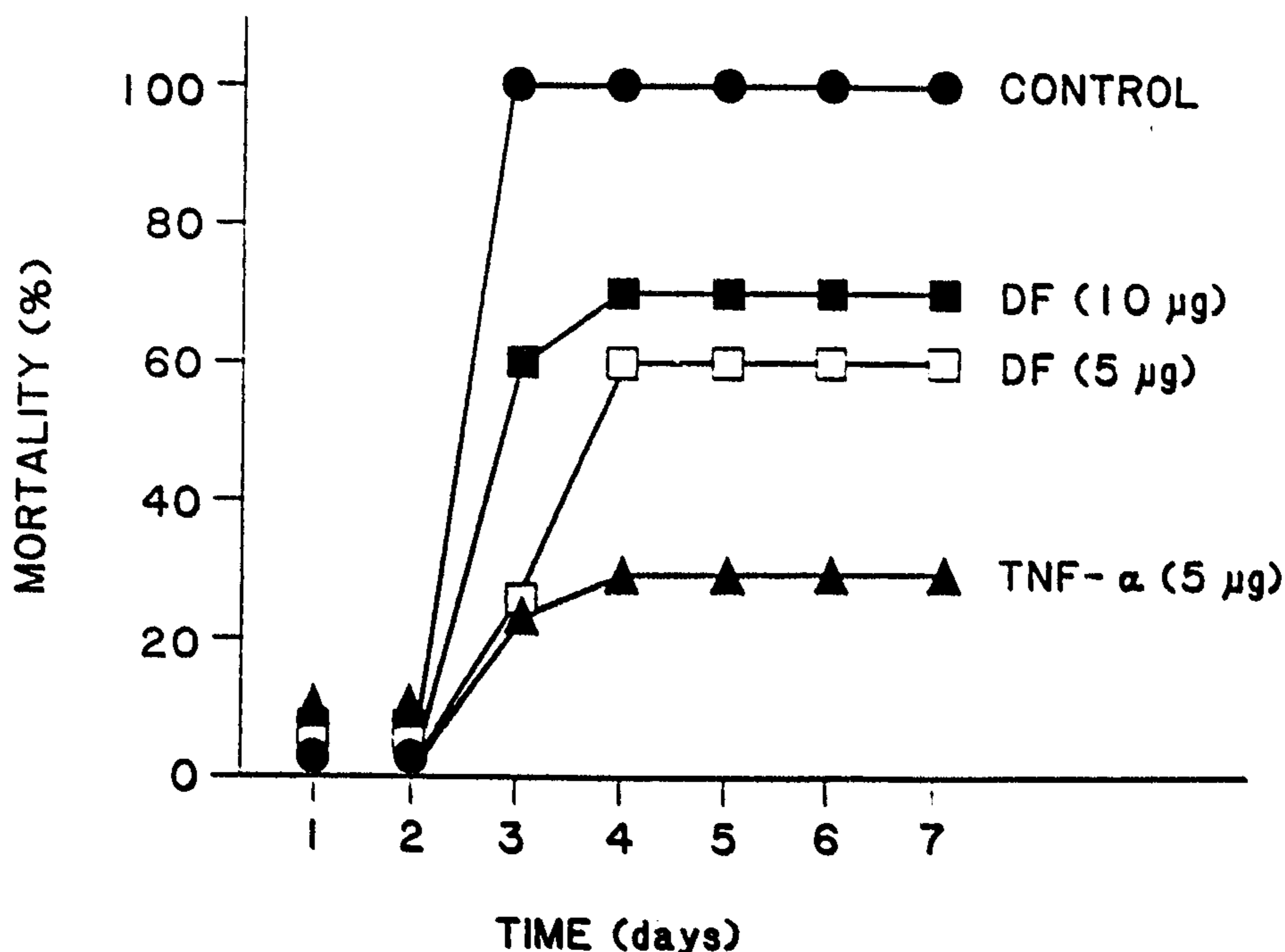
Use of a protective agent(s) selected from the group of tumor necrosis factor - alpha and -beta, growth hormone, IL-1 and D-factor for the production of a composition suitable for protection, inhibition and prevention of the deleterious effects of reactive oxygen species, and administration of an effective amount of the protective agent(s). Treatment of tissues and organs to be transplanted are described. Perfusion solutions and the preparation of perfused, excised tissue are described.



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<p>(21) International Application Number: <b>PCT/US91/07759</b></p> <p>(22) International Filing Date: <b>21 October 1991 (21.10.91)</b></p> <p>(30) Priority data: <b>602,850</b>                      <b>25 October 1990 (25.10.90)</b>      <b>US</b></p> <p>(71) Applicant: <b>GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</b></p> <p>(72) Inventor: <b>WONG, Grace, H., W. ; 324 Commercial Avenue, #5, South San Francisco, CA 94080 (US).</b></p> <p>(74) Agents: <b>ADLER, Carolyn, R. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</b></p>	<p>(81) Designated States: <b>AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</b></p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

## (54) Title: USE OF PROTECTIVE AGENTS AGAINST REACTIVE OXYGEN SPECIES



## (57) Abstract

Use of a protective agent(s) selected from the group of tumor necrosis factor -alpha and -beta, growth hormone, IL-1 and D-factor for the production of a composition suitable for protection, inhibition and prevention of the deleterious effects of reactive oxygen species, and administration of an effective amount of the protective agent(s). Treatment of tissues and organs to be transplanted are described. Perfusion solutions and the preparation of perfused, excised tissue are described.

## Use of protective agents against reactive oxygen species

BACKGROUND OF THE INVENTION

This invention relates to methods for inhibiting, preventing, protecting against or  
5 treating the deleterious effects of reactive oxygen species. The invention also relates to  
methods for protecting against injury to tissues from ischemia and reperfusion injury.

Superoxide radicals ( $O_2^-$ ) and other highly reactive oxygen species such as hydrogen  
peroxide ( $H_2O_2$ ) and hydroxyl radicals (referred to herein as reactive oxygen species or "ROS")  
are produced *in vivo* by enzymatic, spontaneous, and photochemical oxidation reactions.  
10 ROS are produced by mitochondria during electron transport. Other intracellular sources of  
 $O_2^-$  and  $H_2O_2$  are endoplasmic reticulum, peroxisomes, and nuclear and plasma membranes.  
Examples of disorders associated with the generation of ROS include: synovial inflammation  
induced by bacterial lipopolysaccharide endotoxin (LPS), inflammation caused by adjuvant-  
induced arthritis, bleomycin-induced lung fibrosis, reperfusion injury, transplantation rejection,  
15 hyperoxia, and any diseases caused by oxygen and light. It has been suggested that ROS  
may be involved in hyperthermic cell injury (Omar *et al.*, Cancer Res. 47:3473, 1987), and  
that thermosensitivity is linked to a low rate of free radical removal.

Certain agents are capable of inducing superoxide or other oxygen free radicals. Such  
ROS activity may be determined by commonly used methods, such as their ability to induce  
20 guinea pig alveolar macrophages to produce reactive oxygen metabolites which are  
measurable by spectrometer at  $A_{650-640}$  nm (Leurs *et al.*, Biochemistry International 18 (2):295-  
299, 1989), or by known inflammatory or chemiluminescence test models. Agents which  
are known to enhance the production of ROS include but are not limited to the following  
commercially available compounds: inhibitors of glutathione synthesis such as buthionine  
25 sulfoximine, anthracyclines such as adriamycin (doxorubicin), adriamycinone (doxorubicinone),  
daunomycin, daunomycinone, daunorubicin, and daunorubicin derivatives such as 5-  
iminodaunorubicin, ubiquinone, Acid Blues 25, 80, and 41, Acid Green 25, anthraquinone and  
its derivatives such as 2-bromoanthraquinone, 1,2-dihydroxyanthraquinone, 1,8-  
diaminoanthraquinone, 2,6-diaminoanthraquinone, 1,5-dichloroanthraquinone, 1,2-  
30 diaminoanthraquinone, and 2-chloro-anthraquinone, quinizarin, anthrarufin, quinalizarin, aloe-  
emodin and related compounds such as 5-nitro-aloe-emodin, 5-amino-aloe-emodin, 2-allylaloe-  
emodin, averufin, kalafungin, alizarin complexone dihydrate, quercetin dihydrate, acid black  
48, procytoxid, leucotrofina, azimexon, and methoxycin-namonitrile. These ROS inducing  
agents may be administered therapeutically, by intravenous or other methods as desired.

35 A group of metalloproteins known as superoxide dismutases (SOD) catalyze the  
oxidation-reduction reaction  $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$  and thus provide part of the  
defense mechanism against oxygen toxicity. Eukaryotic cells contain copper-zinc SOD, which  
is found predominantly in the cytosol, and MnSOD, which is found mainly in mitochondria.

Extracellular SOD is found primarily in extracellular fluids such as plasma, lymph, and synovial fluid, but occurs also in tissues (Hjalmarsson *et al.*, Proc. Natl. Acad. Sci. USA 84:6340, 1987).

5 The scientific literature suggests that SOD administration may be useful in a wide range of clinical applications. Potential applications include prevention of oncogenesis and of tumor promotion, treatment of inflammations, reduction of the cytotoxic and cardiotoxic effects of anticancer drugs, protection of ischemic tissues and protection of spermatozoa (EPO Appl. EP 0 284 105 A2). It has also been suggested that oxygen free radicals are involved in the pathogenesis of, and that SOD administration protects against, influenza virus infection (Oda  
10 *et al.*, Science 244: 974-976, 1989).

Ischemia causes injury to cells, and if continued for a sufficient length of time, can kill them. Reperfusion after a brief period of ischemia, although beneficial in the long term, frequently results in an initial injury to the tissues upon reoxygenation, presumably through the formation and involvement of reactive oxygen species. This phenomenon has been  
15 described in the literature with heart, skin, intestine, pancreas, and variety of tissues. It is also important to protect against reoxygenation injury during thrombolytic therapy, and in the preservation of organs for transplantation.

D-Factor (hereafter "DF") is a known molecule. It is capable of directing the choice of neurotransmitter phenotype made by cultured rat sympathetic neurons, regulates the growth  
20 and differentiation of embryonic stem cells and myeloid cells and stimulates bone remodeling and acute-phase protein synthesis in hepatocytes. It has been termed DIA, DIF, DRF, HSFIII, human interleukin DA (HILDA) and LIF (leukemia inhibitory factor). This cytokine has been compared to IL-6 and TGF-beta in that it regulates function, growth and differentiation in the embryo and in the adult in many tissues and cell types, including monocytic cells,  
25 megakaryocytes, embryonal stem cells, hepatocytes, adipocytes, osteoblasts and neuronal cells. See Yamamori *et al.*, "Science" 246:1412 (1989), Lowe *et al.*, "DNA" 8(5):351 (1989) and Abe *et al.*, "J. Biol. Chem." 264(15):8941 (1989). DF also is known to induce differentiation of myeloid leukemia cells and therefore has been proposed in the art to be useful in the treatment of myeloid leukemias. DF is currently purified by complex methods  
30 not amenable to large scale commercial development (Hilton *et al.*, "Analyt. Biochem." 173:359 [1988]). The use of DF for protection against injury from radiation or chemotherapy has been disclosed.

Growth hormone (GH) and human growth hormone (hGH) are secreted in the pituitary. The mature form of hGH consists of 191 amino acids and has a molecular weight of about  
35 22,000; its sequence and characteristics are set forth, for example, in Hormone Drugs, Geuriguian *et al.*, U.S.P. Convention, Rockville Md (1982).

hGH has been used for the treatment of hypopituitary dwarfism, and has been proposed for the treatment of burns, wound healing, dystrophy, bone knitting, diffuse gastric

bleeding and pseudarthrosis. The major biological effect of GH is to promote growth. The organ systems affected include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys. Growth hormone exerts its action through interaction with specific receptors on cell membranes. Administration of growth hormone for treatment of pulmonary dysfunction and ventilator dependency has been proposed in PCT WO90/09189 published 23 August 1990; in this application causes of pulmonary dysfunction are stated to include physical injury as well as naturally occurring diseases such as pulmonary obstruction, pneumonia, asthma, emphysema, cancers, autoimmune diseases, neurological disorders including stroke, and chest deformities.

Interleukin-1 (IL-1) is produced by activated macrophages. At least two types exist, designated  $\alpha$  and  $\beta$  (March *et al.*, Nature 315:641-647 (1985)). IL-1 mediates a wide range of biological activities; it has been found to stimulate fibroblast proliferation and to induce in these cells the synthesis of collagenase, prostaglandin E<sub>2</sub> and interferon beta-2, to decrease in adipocytes the activity of lipoprotein lipase, and to activate osteoclasts.

Tumor necrosis factors (TNFs) are polypeptides produced by mitogen-stimulated macrophages (TNF- $\alpha$ ) or lymphocytes (TNF- $\beta$ ) which are cytotoxic to certain malignantly transformed cells but not to certain normal cells (E. A. Carswell *et al.*, Proc. Natl. Acad. Sci. U.S.A. 72:3666, 1975; B. J. Sugarman *et al.*, Science 230:943, 1985; Schultze *et al.*, J. Immunol. 140: 3000, 1988). TNF- $\alpha$  has been suggested to be responsible for wasting and cachexia in patients with cancer or severe infections, and both TNF- $\alpha$  and TNF- $\beta$  mediate many other biological effects. TNF is also known to induce MHC antigens. The inventors herein have reported that TNF induces MnSOD in various transformed and normal cell lines (Wong *et al.*, Science 242:941, 1988).

TNF at certain dosages is known to trigger the generation of ROS in macrophages or neutrophils (Tsujiimoto *et al.*, Biochem. Biophys. Res. Commun. 137:1094, 1986; Matsubara *et al.*, J. Immunol. 137:3295, 1986; Shalaby *et al.*, Leuk. Biol. 41:196, 1987; Berkow *et al.*, J. Immunol. 139:3783, 1987). It has been suggested repeatedly in the literature that TNF at certain dosages enhances tissue injury caused by reactive oxygen species (see, e.g. Clark *et al.*, J. Cell Biochem. Suppl. 12A, p. 40, Jan 1988; Sullivan *et al.*, Infect. and Immunity 56(7): 1722-1729, 1988; and Tiegs *et al.*, Biochem. Pharmacol. 38(4): 627-631, 1989).

The literature has reported that TNF- $\alpha$  and other cytokines such as IL-1 may protect against the deleterious effects of ionizing radiation produced during a course of radiotherapy, such as denaturation of enzymes, lipid peroxidation, and DNA damage (Neta *et al.*, J. Immunol. 136(7):2483, 1987; Neta *et al.*, Lymphokine Res. 5 *et al.*, *supra*; Neta *et al.*, Fed. Proc. 46:1200 (abstract), 1987; Urbaschek *et al.*, Lymphokine Res. 6:179, 1987; U.S. Patent No. 4,861,587; Neta *et al.*, J. Immunol. 140:108, 1988), and that TNF treatment accelerates restoration of hematopoiesis in animals compromised by sublethal doses of cytotoxic drugs or irradiation (Neta, *et al.*, Blood 72(3):1093, 1988). A recent article reported that

pretreatment with TNF protects mice from lethal bacterial infection (Cross *et al.*, J. Exp. Med. 169:2021-2027, 1989). It has also been suggested that administration of subdeleterious amounts of TNF and/or IL-1 may modulate the deleterious effect of subsequent TNF and/or IL-1 administration; this reference further suggests that ionizing radiation may be administered as a sensitizing agent (EPO Appl. EP 0 259 863 A2). It has also been reported that pretreatment of cells with low levels of either TNF or IL-1 can confer resistance to killing by subsequent treatment with TNF- $\alpha$  and cycloheximide in combination (Wallach, J. Immunol. 132:2464, 1984; Hahn *et al.*, Proc. Natl. Acad. Sci. U.S.A. 82:3814, 1985; Holtmann *et al.*, J. Immunol 139:1161, 1987). It has been suggested that inadequate endogenous levels of TNF may be involved in the development of diabetes and of lupus erythematosus (Jacob *et al.*, Nature 331:356-358, 1988).

Saez *et al.*, Proc. Natl. Acad. Sci. USA 84: 3056-3059, 1987, have shown that SOD protects cultured neurons against death by glucose deprivation. Tsan *et al.*, J. Appl. Physiol. 68(3): 1211-1219, 1990, has recently confirmed that tracheal insufflation of TNF (and IL-1 in unpublished data) protects rats against oxygen toxicity.

White *et al.*, J. Clin. Invest, 79: 1868-1873, 1987, have shown that pretreatment with a combination of TNF and IL-1 decreases lung injury and mortality in rats exposed to hyperoxia, but indicate that, with i.p. injection, either TNF or IL-1 alone protected the rats only comparable to saline.

It is an object of this invention to provide methods for protecting ischemic tissues, such as those tissues, bones or organs to be transplanted from a donor to a recipient patient, or those tissues whose oxygen supply has been blocked, from the effects of ROS.

It is a further object herein to provide methods to inhibit, prevent or treat reperfusion injury, bronchopulmonary dysplasia, stroke, arteriosclerosis, atherosclerosis, myocardial infarct, inflammatory autoimmune diseases, viral infection, inflammation-induced arthritis, hyperoxia, sepsis, diabetes, influenza, multiple sclerosis, hyperbaric treatment after premature birth, acquired immunodeficiency syndrome, transplant rejection or transplantation injury, bleomycin-induced lung fibrosis, synovial inflammation induced by bacterial LPS endotoxin, lung injury resulting from immune complexes, hyperbaric treatment of cancer, kidney disease, Parkinson's disease, sickle cell anemia, sickle cell trait, alcoholic or non-alcoholic cirrhosis, or other diseases associated with toxic ROS.

Another object of this invention is to supply perfusion solutions and excised, perfused tissues for transplant.

These and other objects of the invention will be apparent from consideration of the specification as a whole.

#### SUMMARY OF THE INVENTION

Unexpectedly, and contrary to the suggestions in the literature, the present inventors have discovered that treatment with certain doses of TNF and/or IL-1, alone or in combination

with growth hormone and/or D-factor, protect against ROS damage.

The objects of this invention are accomplished by methods utilizing these effects of the protective agent(s) TNF, IL-1, GH and DF. In one preferred method, a patient at risk for reperfusion injury is treated with an effective but sub-deleterious amount of at least one of these agents before, at, or after, but preferably prior to reperfusion. In another embodiment, a patient with a condition or disease associated with ROS (as described above) is treated with a dose of at least one of these agents which is effective at preventing, inhibiting or treating that condition.

Other preferred embodiments relate to tissue transplant. In one embodiment, ischemic tissue is protected by the administration of an effective amount of at least one of TNF, GH, DF, and/or IL-1 prior to reperfusion of the tissue. This ischemic tissue may be treated prior to or after removal from a donor, and before or after it is transferred to or implanted in a recipient patient.

This invention also encompasses perfusion solutions comprising at least one of the above protective agent(s) in a pharmaceutically acceptable excipient, as well as perfused, excised tissues, where the tissues are perfused with such a perfusion solution.

In an alternate embodiment, the protective agent(s) is administered to a tissue donor prior to preparation for removal of the tissue.

#### DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows that TNF pretreatment protects against heart damage due to ischemia and reperfusion.

FIGURE 2 shows the induction of MnSOD in ischemic rat hearts pretreated with TNF prior to reperfusion.

FIGURE 3 depicts the mature amino acid sequences for human (H) (Sequence ID No. 1) and murine (M) (Sequence ID No. 2) D-factor. Areas of homology are boxed.

FIGURE 4A illustrates the results obtained in Example 2, showing that TNF- $\alpha$  and DF protect adult rats from oxygen toxicity.

FIGURE 4B shows the synergistic effect of DF plus TNF.

FIGURE 5 illustrates the results obtained in Example 2, showing that TNF- $\alpha$  and GH protect adult rats from oxygen toxicity.

FIGURE 6 shows the protective effect of TNF on neuronal survival after glucose deprivation. The number of neurons dying under each condition (10,000 total neurons per dish) are shown.

#### DETAILED DESCRIPTION

Tumor necrosis factor or TNF, as employed herein, refers in general to the various forms of TNF which exhibit one or more biologic properties of tumor necrosis such as tumor cell lysis, inhibition of infectious agents, MHC antigen induction, and neutralization by antibody to TNF- $\alpha$  or TNF- $\beta$  but not by antibodies to other cytokines. It is believed that

gamma interferon is synergistic with TNF in anti-tumor or anti-viral assays for TNF, and may therefore be desirably administered along with TNF in the practice of this invention.

In particular, the tumor necrosis factors useful herein include TNF- $\alpha$  and TNF- $\beta$ . The former is described in copending EPO Appl. EP 0 168 214 A2 together with methods for its synthesis in recombinant cell culture. Similarly, the latter (previously called lymphotoxin) and suitable recombinant synthesis methods are described in copending EPO Appl. EP 0 164 965 A2. The TNF- $\alpha$  and TNF- $\beta$  described in these applications include cytotoxic amino acid sequence and glycosylation variants which also are used herein. Of course, TNF- $\alpha$  or TNF- $\beta$  from non-recombinant sources are also useful in the practice of this invention.

TNF- $\alpha$  or TNF- $\beta$  are used alone or in admixture with one another in proportions empirically determined to exert the most effective clinical response. TNF is not species specific, so TNFs from other animal species, e.g. porcine or bovine, are useful for treatment of humans. The preferred TNF for treatment of humans is mature human TNF- $\alpha$  from recombinant microbial cell culture. The TNF ordinarily will have a cytolytic activity on susceptible L-929 murine cells of greater than about  $1 \times 10^6$  units/mg, wherein a unit is defined as set forth in the above-described patent applications.

As used herein, the terms "growth hormone" or "GH" denote growth hormone produced from natural source extraction and purification, as well as by recombinant cell culture systems. "hGH" refers to human growth hormone. See, for example, U.S. Patent 4,321,832. The terms likewise cover biologically active human growth hormone equivalents, e.g., those differing by one or more amino acids(s) in the overall sequence. Further, the terms as used in this application are intended to cover substitution, deletion and insertion amino acid variants of GH, or post-translational modifications.

As used herein, "IL-1" denotes interleukin-1 produced from natural source extraction and purification, as well as by recombinant cell culture systems. See, for example, March *et al.*, Nature 315:641-646 (1985)). The terms likewise cover biologically active IL-1 equivalents, e.g., those differing by one or more amino acids(s) in the overall sequence. Further, the terms as used in this application are intended to cover substitution, deletion and insertion amino acid variants of IL-1, or post-translational modifications.

The mature amino acid sequences for human (H) and murine (M) DF shown in Figure 3 are disclosed in EP 285,448, published 5 Oct. 1988, especially at Fig. 26, including methods for its production in recombinant cell culture. See also D. P. Gearing *et al.*, Nucleic Acids Res. 16:9857 (1988), and N. M. Gough *et al.*, Proc. Nat. Acad. Sci. USA 85:2623-2627 (1988). For purposes of this application, DF is defined herein as any polypeptide having (a) cytoprotective activity as defined below and (b) amino acid sequence which is homologous to either amino acid sequence of Figure 3.

Homologous, as to DF for example, for the purposes herein means that the candidate polypeptide comprises the epitope of DF that is functional in conferring protection on

experimental animals, or functionally conserved amino acid variants thereof. Identification of this epitope is a matter of routine experimentation. Most typically, one would conduct systematic substitutional mutagenesis of the DF molecule while observing for reductions or elimination of cytoprotective activity. This is commonly accomplished by sequentially substituting each residue of the native DF sequence with alanine. Preferably, the residues to be substituted will be those which are (1) identically conserved among animal species, (2) located on disulfide bonded loops, and (3) hydrophilic in nature. Domains containing more than 4 identically conserved residues among animal species and which are not found within about 3 residues of a site of glycosylation are to be preferred for mutagenesis. Preferred regions for mutagenesis are 12-25, 34-60 and 121-143 of the Figure 3 sequences. In any case, it will be appreciated that due to the size of DF most substitutions will have no effect on DF activity, but if some effect is seen it will be modestly agonistic or antagonistic. The great majority of DF variants will possess at least some cytoprotective activity, particularly if the substitution is conservative. Conservative substitutions are substitutions from the same classes, defined as acidic (Asp, Glu), hydroxy-like (Cys, Ser, Thr), amides (Asn, Gln), basic (His, Lys, Arg), aliphatic-like (Met, Ile, Leu, Val, Gly, Ala, Pro), and aromatic (Phe, Tyr, Trp). The 9 C-terminal hydrophobic residues should only be substituted, if at all, with other hydrophobic residues. The first 3 N-terminal residues may be deleted or freely substituted, and may include a serine residue inserted at the N-terminus.

Alternatively, or in addition, the active site is identified by raising antibodies against the intact native DF, screening for neutralizing antibodies, and determining the site to which the neutralizing antibodies bind. Neutralizing antibodies against DF also would find use in diagnostic immunoassays for DF, especially when used in a sandwich-type immunoassay in concert with a non-neutralizing antibody directed against another DF epitope.

Homologous sequences generally will be greater than about 30 percent homologous on an identical amino acid basis, ignoring for the purposes of determining homology any insertions or deletions from the candidate molecule in relation to either native sequence of Figure 3. Homologies of about 50, 70 and 90% are also included within the scope hereof so long as the molecules possess the requisite cytoprotective activity.

The cytoprotective agents discussed herein, e.g. DF, GH, TNF, IL-1, also includes glycosylation variants as well as unglycosylated forms of the agents, fusions of the agents with heterologous polypeptides, and fragments of the agents, again so long as the variants possess the requisite cytoprotective activity.

The formulations may contain compounds previously suggested for use in the treatment of the conditions and diseases described herein, as well as those compounds previously suggested for use in preventing damage from ROS. Antioxidants, such as ascorbate, fibrinolytic agents such as tissue plasminogen activator, and other compounds previously suggested for use in preventing reperfusion injury may also be included. Agents

that block the toxicity of high doses of TNF and/or IL-1--such as glucocorticoids and indomethacin--without altering the MnSOD-inducing activity of TNF--are also utilized in the practice of this invention. Agents which block induction of MHC antigens are also desirably administered. Compounds which effect the redox potential of ROS may also be utilized in a formulation. The protective agent(s) also is suitably formulated together with known agents in order to modify or enhance half-life or therapeutic activity. These other agents or therapies are used at the same time as the TNF, IL-1, GH and/or DF is administered or in a sequential course of therapy.

The TNF, DF, GH, and/or IL-1 are placed into sterile, isotonic formulations together with required cofactors. Formulations may contain one or more protective agents. The formulations are preferably liquid, and ordinarily a physiologic salt solution or dextrose solution, together with conventional stabilizers and/or incipients. Compositions may also be provided as lyophilized powder for ultimate delivery in solution. Saline is a suitable carrier, although other conventional parenteral solutions or buffers are usable.

In a pharmacologic sense, in the context of the present invention, a therapeutically effective amount of TNF, DF, GH, and/or IL-1 refers to that amount effective to protect normal cells from the deleterious effects of ROS. It has been discovered that TNF and/or IL-1 plus GH, and TNF and/or IL-1 plus DF provide synergistic protection against oxygen free-radical damage such as caused by hyperoxia, and thus relatively smaller dosages of a particular agent may be administered (See Figs. 4 and 5).

Although it is currently believed that induction of MnSOD is not sufficient to protect cells from damage, monitoring of its induction by TNF is a convenient indication of TNF activity under this invention. As shown in Fig. 2, TNF induces MnSOD in ischemic rat hearts pretreated with TNF before reperfusion.

The therapeutically effective dosage of TNF to be administered to a human patient or human tissue generally will range from about 1-250  $\mu\text{g}/\text{m}^2$  per dose, and preferably from about 1-10  $\mu\text{g}/\text{m}^2$ , and most preferably 10  $\mu\text{g}/\text{m}^2$ , although the dose of the TNF administered will be dependent upon the species of the patient, the properties of the TNF employed, e.g. its activity and biological half-life, the concentration of the TNF in the formulation, the rate of dosage, the clinical tolerance of the patients involved, the pathological condition of the patients and the like, as is well within the skill of the physician. It will be appreciated that the practitioner will adjust the therapeutic dose in line with clinical experience for any given TNF. Preferably, the TNF is administered intravenously or intramuscularly.

The amount of DF, GH, TNF and/or IL-1 which is used will depend upon the therapeutic protocol, considering the use of hyperoxia, irradiation or chemotherapy, the condition of the patient, the activity of the agent used, the administration route, and other influences that will be appreciated by the ordinary artisan. In mice, a dosage of about 7.5 micrograms of DF ip/mouse is acceptable, with about 0.5 micrograms to 20 micrograms per day being the range

from the lowest level that is marginally effective for cytoprotection to a safe and adequate upper limit. Obviously, the dose will differ for other animals and humans and will vary depending upon administration routes. Treatment effective amounts of IL-1 administered to mice range between about 0.5 to 25 nanograms per gram of body weight of mouse.

5 For hGH, a suitable dosage for human administration ranges from 0.001 mg per kg of bodyweight per day to about 0.2 mg per kg of bodyweight per day. Generally, daily dosages of GH will be from about 0.05 mg per kg of bodyweight per day. Normally, from 0.07 to 0.15 mg/kg, in one or more applications per day, is effective to obtain the desired result. In an alternative approach, the GH, particularly where formulated in a timed-release form, may  
10 be administered less frequently, i.e., every other day or every third day for certain indications. It is presently preferred the GH be administered within 0 to 24 hours following exposure to free-radical injury.

In the practice of this invention, compositions which include a therapeutically effective amount of TNF, DF, GH, and/or IL-1 are administered to patients having or at risk for damage  
15 from ROS. Accordingly, in some embodiments of this invention, the protective agent, alone or with other agents as described above, is administered to a patient within a temporal period, most preferably within 24 hours prior, preferably prior to or concurrent with, or shortly following exposure, to ROS. In the case of reperfusion, exposure to the ROS may be predetermined and deliberate, or may be a consequence of other therapeutic measures.

20 In other embodiments, a patient's ROS exposure is of a more chronic or regular nature, as with certain of the diseases and chronic conditions described above. In these situations, the protective agent(s) may be administered as part of a long-term course of therapy.

Some embodiments deal with the transfer to and implantation of tissues, and the complex tissues known as organs, in a recipient patient. For purposes of this invention, the  
25 term "tissues" shall be understood to include, without limitation, muscle tissue, connective tissue, epithelial tissue, nervous tissue, vascular tissue, bone, brain, reproductive organs, respiratory organs, digestive organs, excretory organs, urinary organs, sensory organs, and skeletal muscle organs. Particularly preferred tissues include heart, lung, kidney, liver, skin and bone grafts. Suitable tissues are synthetic as well as those which are removed from a  
30 donor.

For tissues to be removed from a donor prior to their transfer to and implantation in a patient, treatment with TNF, GH, DF, and/or IL-1 may be accomplished in several different ways. The protective agent(s) may be administered to the donor, as described above. Alternatively, the tissue itself may be treated, either prior to or after removal from the donor,  
35 and either prior to or after transfer to the patient, but most preferably prior to or concurrent with reperfusion.

Since the first successful human orthotopic liver transplant by Starzl in 1967, methods for the transplant of tissues, as well as suitable protocols for their perfusion with various

agents, have become commonly known in the art. Suitable protocols and perfusion solutions are described, for example, in Kalayoglu *et al.*, *The Lancet*, March 19, 1988, pp. 617-619. Perfusion is commonly accomplished with a mechanical pump, as described in Example 1 below. Suitable perfusion solutions include lactated Ringer's solution, UW solution, and pharmaceutically acceptable isotonic solutions. These solutions enable a TNF, DF, GH, and/or IL-1 -treated tissue to be preserved by continuous perfusion or cold storage until it is implanted into a recipient patient.

In an embodiment of this invention, perfusion solutions are provided, comprising TNF, DF, GH and/or IL-1 at concentrations of approximately 1 - 250  $\mu\text{g}/\text{m}^2$  with a pharmaceutically acceptable isotonic solution.

For each type of type of disease or injury, and for each animal species to be treated, the exact protective TNF, DF, GH, and/or IL-1 dosage necessary for this protective effect may vary from that shown herein, and the exact administration parameters suitable for any given patient or animal species will be determined by routine experimentation. Typically, the patient or tissue is first administered the agent, e.g. by intravenous or intramuscular administration, and thereafter exposed to ROS. The patient or tissue is monitored for symptoms of any beneficial or deleterious effects of the treatment. If the initial treatment is partially successful or unsuccessful, the process may be repeated, optionally with a modification of the dosage or route of administration.

According to this invention, patients from differing species are all treated by the pharmaceutically acceptable administration of TNF, DF, GH, and/or IL-1 in a pharmaceutically effective dosage and for a period of time sufficient to inhibit, prevent, protect from the damaging effects of ROS.

It is also envisioned that, in the practice of this invention, administration of the protective agent(s) may be accompanied by the therapeutic administration of a course of radiation, heat, and/or ROS inducing agents. The agent, alone or with other agents, may be administered to a patient prior to, following, or simultaneously with exposure to radiation, heat, or ROS inducing agents. Exposure to the radiation, heat, or ROS inducing agents may be predetermined and deliberate, or may be a consequence of other therapeutic measures. Heat or ROS-inducing agents may be administered with radiation, and/or with an additional dose of TNF, DF, GH and/or IL-1.

ROS inducing agents and their methods of administration are described above. Radiation and heat are administered by protocols commonly known to practitioners, as described in the literature cited above. Typically, radiation is given in pulses over a period of time from 1 - 8 weeks, for a total dose of approximately 1000-1200 rads. Heat may be administered by known methods such as a heat blanket or hyperthermia chamber, for a period sufficient to raise a patient's body tissue temperature above 37 °C, preferably 40 - 45 °C, and most preferably to approximately 42 °C.

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Where GH alone is administered to a patient having or at risk of hyperoxia damage, the patient is may be subjected to atmospheric conditions with greater levels of oxygen than normal atmospheric conditions.

5 In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only, and are not to be construed as limiting this invention in any manner.

#### EXAMPLE 1

##### The effect of TNF on isolated rat heart.

10 This experiment was conducted by Dr. Lynn Eddy at the University of Southern California. Healthy Sprague Dawley rats weighing between 200 and 300g are anesthetized with sodium pentobarbital, 30 mg/kg, intraperitoneally. Heparin (500 U) is administered intravenously. The chests are opened and the hearts rapidly excised and placed in iced cold buffer until they stop beating. The hearts are mounted by the aortic roots to a stainless steel  
15 cannula and perfused through the aorta in a retrograde manner (Langendorff). Krebs-Henseleit buffer of the following composition is used throughout the experiments (final concentration in mM/L): NaCl, 118; KCl, 4.7; CaCl, 2.5; MgSO, 1.2; KH PO, 1.2; CaEDTA, 0.5; NaHCO, 25; and glucose, 10. The buffer is aerated with 95% O - 5% CO. Perfusion is maintained by a peristaltic pump at a flow rate of 10 ml/min and perfusion pressure is  
20 monitored by a P23AA Statham\*pressure transducer attached to a side arm of the aortic inflow cannula and recorded on a Hewlett Packard 7702B recorder. The complete perfusion system is maintained within a thermostatically controlled plexiglass chamber maintained at 37°C.

The hearts are perfused 3-4 minutes to remove blood. After a 20 minute control  
25 period, global ischemia is initiated by stopping the pump and turning off the 95% O - 5% CO. At the end of the ischemic period, the pump is restarted and the buffer reoxygenated.

Enzyme leakage from the heart cells is determined in effluent samples collected from the heart at specific time periods during the control period and during reperfusion. Lactic dehydrogenase (LDH) activity is assayed by monitoring the oxidation of NADH, using pyruvate  
30 as the substrate. NADH is monitored at 340 nm using a Perkin Elmer Lambda 3\*recording spectrophotometer. As shown in Fig. 1, LDH activity from hearts treated with 10µg TNF compared to controls indicates that the TNF protected the heart from damage mediated by its ischemia and reperfusion.

#### EXAMPLE 2

35 This experiment was conducted by Dr. Min-Fu Tsan, at the Department of Veterans Affairs Medical Center in Albany, New York. The effects of TNF-alpha, TNF-beta, IL-1 beta, interferon (IFN)-gamma, growth hormone and DF, alone and in various combinations, on the protection of rats against oxygen toxicity was examined.

\*-trademarks

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Tracheal insufflation of the cytoprotective agent and the exposure of the rats to hyperoxia was performed essentially as described in Tsan *et al.*, *J. Appl. Physiol.* 68: 1211-1219, 1990. Briefly, male Sprague-Dawley rats (Harlan Sprague Dawley, Altamont, NY, USA) free of respiratory infections and weighing between 250-350 g, were anesthetized with methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ, USA) and were intubated with a 16 G i.v. catheter (Angiocath, Becton Dickinson, Sandy, Utah, USA). One ml of the protective agent(s) (agents and concentrations are shown in the next paragraph) was administered to the rats in calcium-magnesium free Hanks' balanced salt solution (HBSS) (or 1 ml HBSS for control animals) followed by 2 ml of air, injected through the intratracheal catheter. Auscultation of the chest with a stethoscope was done to insure that the agent was insufflated into the lungs. After the rats had recovered from the effect of anesthesia, they were placed in groups of 5 in a lucite chamber (45 by 40 by 25 cm<sup>3</sup>) which was flushed with 100 % O<sub>2</sub> at 15 liter/min for 5 min and then maintained at 5 liter/min. The concentration of O<sub>2</sub> in the chamber as monitored using an oxygen analyzer (Hudson Oxygen Analyzer,\* Ventronics Products Division, Temecula, CA, USA) was more than 95 % at all times. The rats were given free access to water and diet. Control exposure (normoxia) was performed in room air.

Results are shown in Figures 4 and 5, and the percent survival after 3 days and 7 days are shown below.

Table 1

Tracheal insufflate	n	3 days	7 days
HBSS (control)	45	0	0
DF 5 $\mu$ g	10	70	40
DF 10 $\mu$ g	10	40	30
GH 5 $\mu$ g	15	53	27
GH 10 $\mu$ g	10	50	40
TNF- $\alpha$ 5 $\mu$ g	17	76	71
TNF- $\alpha$ 1 $\mu$ g	10	0	0
DF 5 $\mu$ g + TNF 1 $\mu$ g	15	100	93
GH 5 $\mu$ g + TNF 1 $\mu$ g	15	67	67
	*	*	*

In data not shown, IL-1 was also protective comparable to TNF. It may be seen from these results that TNF or IL-1 alone are effective in preventing death due to hyperoxia. Growth Hormone and DF are less effective for the same purpose when used alone; but synergize with TNF and/or IL-1 in protecting the animals. This work indicates that these agents be used in any hyperbaric treatment, including oxygen treatment of premature infants and cancer patients.

\*-trademark

Example 3

This experiment was conducted by Dr. John A. Kessler, at the Albert Einstein College of Medicine of Yeshiva University, New York New York. The protective effect of TNF on neuronal survival after glucose deprivation (analogous to nutrient depletion following ischemia) was studied according to the method of Saez *et al.*, Proc. Natl. Acad. Sci. USA 84: 3056-3059, 1987. As shown in Fig. 6, TNF was found to give a strongly protective effect; maximal effects are shown at 1 ng/ml of TNF.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: GENENTECH, INC.
- (ii) TITLE OF INVENTION: Method for Protection Against Reactive Oxygen Species
- 10 (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Genentech, Inc.  
 (B) STREET: 460 Point San Bruno Blvd  
 15 (C) CITY: South San Francisco  
 (D) STATE: California  
 (E) COUNTRY: USA  
 (F) ZIP: 94080
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: patin (Genentech)
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:  
 (B) FILING DATE: 21-OCT-1991  
 (C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: USSN 07/602850  
 (B) FILING DATE: 25-OCT-1990
- 35 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Adler, Carolyn R.  
 (B) REGISTRATION NUMBER: 32,324  
 (C) REFERENCE/DOCKET NUMBER: 605P1
- 40 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 415/266-2614  
 (B) TELEFAX: 415/952-9881  
 (C) TELEX: 910/371-7168

## 45 (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 179 amino acids  
 (B) TYPE: amino acid  
 50 (D) TOPOLOGY: linear

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

55 Pro Leu Pro Ile Thr Pro Val Asn Ala Thr Cys Ala Ile Arg His  
 1 5 10 15

Pro Cys His Asn Asn Leu Met Asn Gln Ile Arg Ser Gln Leu Ala  
 20 25 30

60 Gln Leu Asn Gly Ser Ala Asn Ala Leu Phe Ile Leu Tyr Tyr Thr  
 35 40 45

Ala Gln Gly Glu Pro Phe Pro Asn Asn Leu Asp Lys Leu Cys Gly  
 50 55 60

65 Pro Asn Val Thr Asp Phe Pro Pro Phe His Ala Asn Gly Thr Glu  
 65 70 75

Lys Ala Lys Leu Val Glu Leu Tyr Arg Ile Val Val Tyr Leu Gly  
 80 85 90

5 Thr Ser Leu Gly Asn Ile Thr Arg Asp Gln Lys Ile Leu Asn Pro  
 95 100 105

Ser Ala Leu Ser Leu His Ser Lys Leu Asn Ala Thr Ala Asp Ile  
 110 115 120

10 Leu Arg Gly Leu Leu Ser Asn Val Leu Cys Arg Leu Cys Ser Lys  
 125 130 135

Tyr His Val Gly His Val Asp Val Thr Tyr Gly Pro Asp Thr Ser  
 140 145 150

15 Gly Lys Asp Val Phe Gln Lys Lys Lys Leu Gly Cys Gln Leu Leu  
 155 160 165

20 Gly Lys Tyr Lys Gln Ile Ile Ala Val Leu Ala Gln Ala Phe  
 170 175 179

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 25 (A) LENGTH: 179 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

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

30 Pro Leu Pro Ile Thr Pro Val Asn Ala Thr Cys Ala Ile Arg His  
 1 5 10 15

35 Pro Cys His Gly Asn Leu Met Asn Gln Ile Lys Asn Gln Leu Ala  
 20 25 30

Gln Leu Asn Gly Ser Ala Asn Ala Leu Phe Ile Ser Tyr Tyr Thr  
 35 40 45

40 Ala Gln Gly Glu Pro Phe Pro Asn Asn Val Glu Lys Leu Cys Ala  
 50 55 60

Pro Asn Met Thr Asp Phe Pro Ser Phe His Gly Asn Gly Thr Glu  
 65 70 75

45 Lys Thr Lys Leu Val Glu Leu Tyr Arg Met Val Ala Tyr Leu Ser  
 80 85 90

50 Ala Ser Leu Thr Asn Ile Thr Arg Asp Gln Lys Val Leu Asn Pro  
 95 100 105

Thr Ala Val Ser Leu Gln Val Lys Leu Asn Ala Thr Ile Asp Val  
 110 115 120

55 Met Arg Gly Leu Leu Ser Asn Val Leu Cys Arg Leu Cys Asn Lys  
 125 130 135

Tyr Arg Val Gly His Val Asp Val Pro Pro Val Pro Asp His Ser  
 140 145 150

60 Asp Lys Glu Ala Phe Gln Arg Lys Lys Leu Gly Cys Gln Leu Leu  
 155 160 165

65 Gly Thr Tyr Lys Gln Val Ile Ser Val Val Val Gln Ala Phe  
 170 175 179

## CLAIMS

1. Use of a protective agent in the production of a composition for the prophylaxis and/or treatment of the deleterious effects of reactive oxygen species in a patient, said protective agent being selected from the group of:
  - (a) growth hormone ('GH')
  - (b) tumor necrosis factor ('TNF') and GH
  - (c) interleukin-1 ("IL-1") and GH, and
  - (d) IL-1 and TNF and GH.
2. The use according to claim 1, wherein said patient has or is at risk of having reperfusion injury, bronchopulmonary dysplasia, stroke, arteriosclerosis, atherosclerosis, myocardial infarct, sepsis, acquired immunodeficiency syndrome, diabetes, multiple sclerosis, inflammation-induced arthritis, hyperbaric treatment of premature birth, hyperoxia, transplant rejection or transplantation injury, sickle cell anaemia, sickle cell trait, hyperbaric cancer treatment, alcoholic or non-alcoholic cirrhosis, bleomycin-induced lung fibrosis, synovial inflammation induced by bacterial LPS endotoxin, lung injury resulting from immune complexes, or Parkinson's disease.
3. The use according to claim 1 or claim 2, wherein said tumor necrosis factor is tumor necrosis factor- $\alpha$  or tumor necrosis factor- $\beta$ .
4. The use according to claim 1, wherein said composition also comprises a fibrinolytic agent or antioxidant.
5. The use of a protective agent selected from those set out in claim 1, in the production of a composition suitable for the protection of ischemic tissues.
6. The use according to claim 5, wherein said tumor necrosis factor is tumor necrosis factor- $\alpha$  or tumor necrosis factor- $\beta$ .

7. The use according to claim 5, wherein said composition is formulated for perfusion into said tissue.
8. The use according to claim 5, wherein said composition also comprises a pharmaceutically acceptable excipient.
9. The use according to claim 5, wherein said composition further comprises an antioxidant or fibrinolytic agent.
10. The use according to claim 5, wherein said tissue is selected from the group consisting of muscle tissue, connective tissue, epithelial tissue, nervous tissue, vascular tissue, bone, brain, reproductive organs, respiratory organs, digestive organs, excretory organs, urinary organs, sensory organs, and skeletal muscle organs.
11. Use of a protective agent selected from the group consists of:
  - (a) growth hormone ('GH')
  - (b) tumor necrosis factor ('TNF') and GH
  - (c) interleukin-1 ("IL-1") and GH, and
  - (d) IL-1 and TNF and GHfor the prophylaxis and/or treatment of the deleterious effects of reactive oxygen species in a patient.
12. The use according to claim 11, wherein said patient has or is at risk of having reperfusion injury, bronchopulmonary dysplasia, stroke, arteriolosclerosis, atherosclerosis, myocardial infarct, sepsis, acquired immunodeficiency syndrome, diabetes, multiple sclerosis, inflammation-induced arthritis, hyperbaric treatment of premature birth, hyperoxia, transplant rejection or transplantation injury, sickle cell anaemia, sickle cell trait, hyperbaric cancer treatment, alcoholic or non-alcoholic cirrhosis, bleomycin-induced lung fibrosis, synovial inflammation induced by

bacterial LPS endotoxin, lung injury resulting from immune complexes, or Parkinson's disease.

13. The use according to claim 11 or claim 12, wherein said tumor necrosis factor is tumor necrosis factor- $\alpha$  or tumor necrosis factor- $\beta$ .

14. The use according to claim 11 to 13, wherein said composition also comprises a fibrinolytic agent or antioxidant.

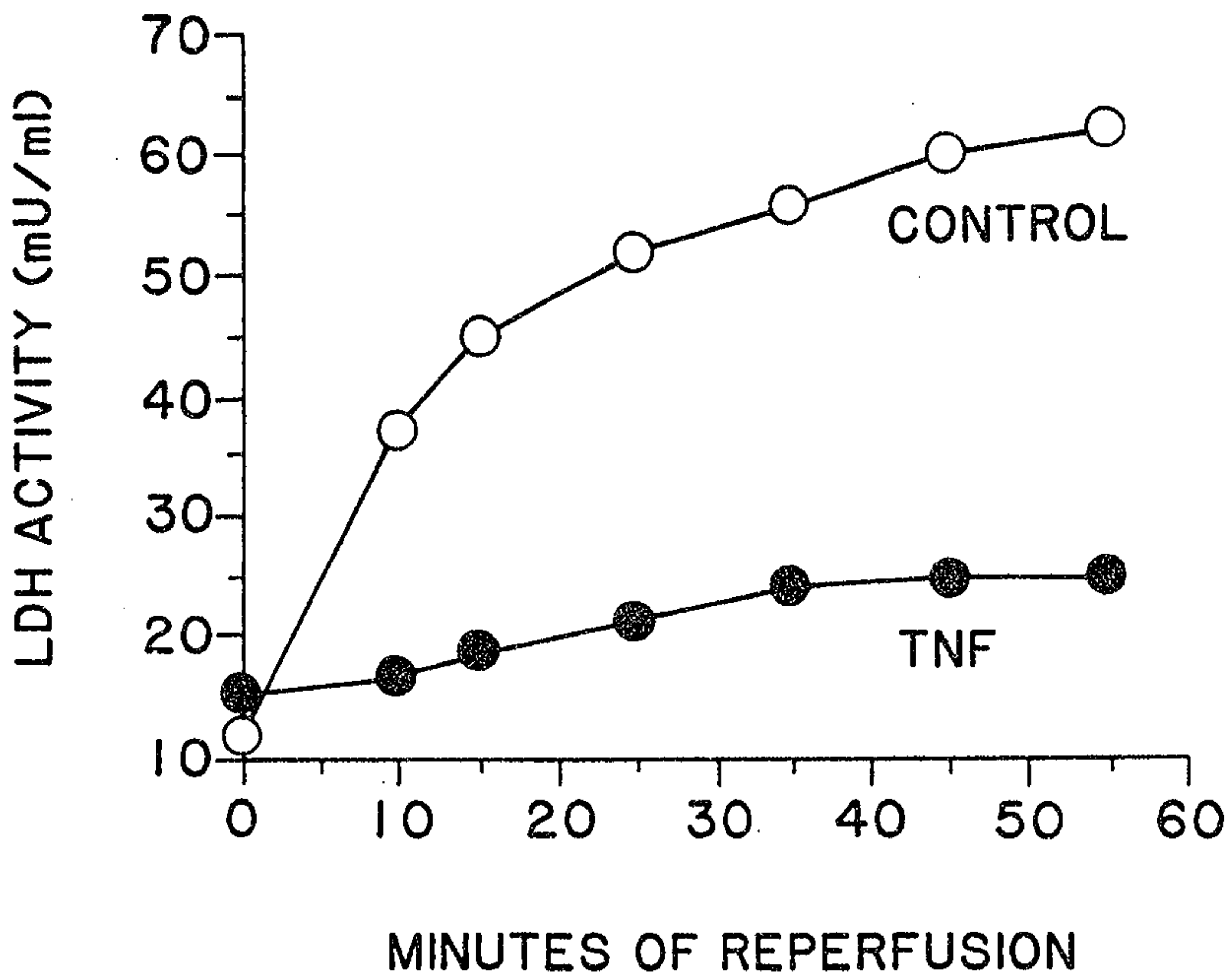


FIG. 1

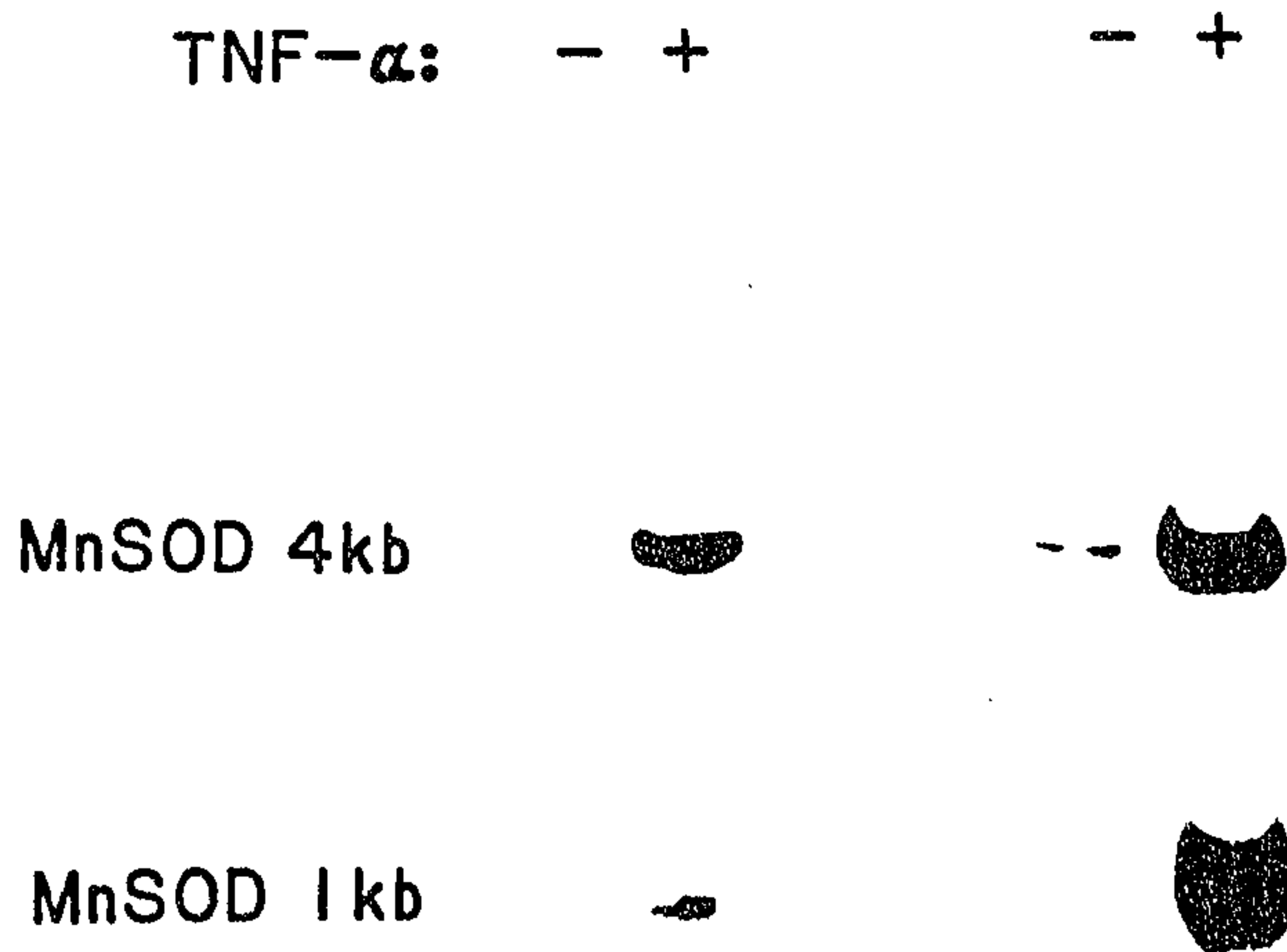
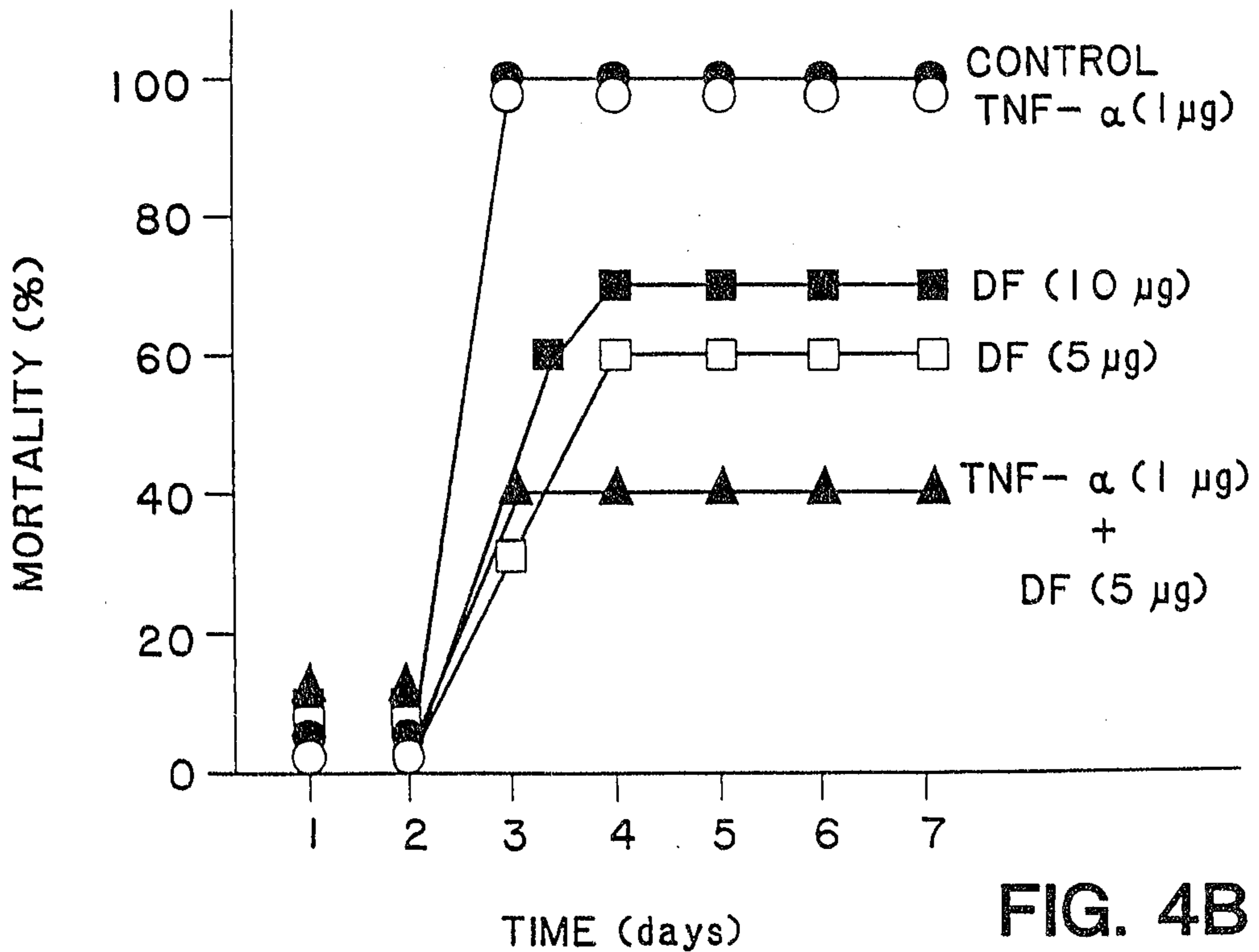
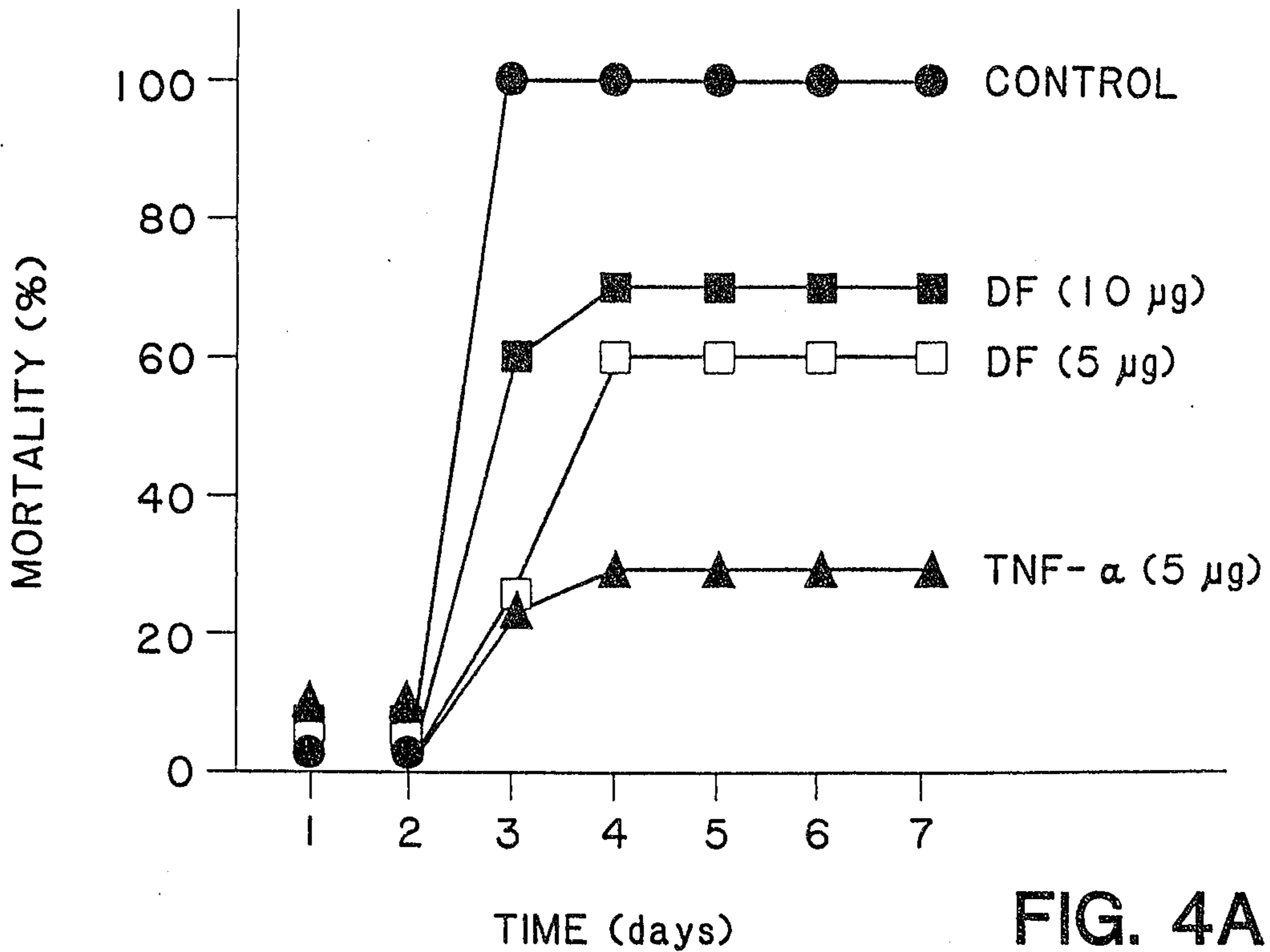


FIG. 2

hdf	1	PLPITPVNATCAIRHPCH	NLMNQIRSN	QLAQLNGSANA	LFI	LYYTAQGE	P
mdf	1	PLPITPVNATCAIRHPCH	GNLMNQIKN	QLAQLNGSANA	LFI	LYYTAQGE	P
hdf	51	FPNNLDKLCGPNVTDFP	FHANGTEK	AKLVELYR	IVVYLGTS	LGNITRD	Q
mdf	51	FPNNVEKLCAPNMTDFP	FHANGTEK	AKLVELYR	IVVYLGTS	LGNITRD	Q
hdf	101	KILNPSALSLSHSKLNAT	ADILRGLLSNVLC	RLCS	KYHVG	HVDVTY	GPDT
mdf	101	KVLNPTAVSLQVSKLNAT	IDVMRGLLSNVLC	RLCS	KYRV	HVDVPP	PDHS
hdf	151	GKDVFFQK	KKLGCQLL	GK	YKQ	IIAV	LQAF
mdf	151	DKEAFFQR	KKLGCQLL	GT	YKQ	VIS	VVQAF

FIG. 3

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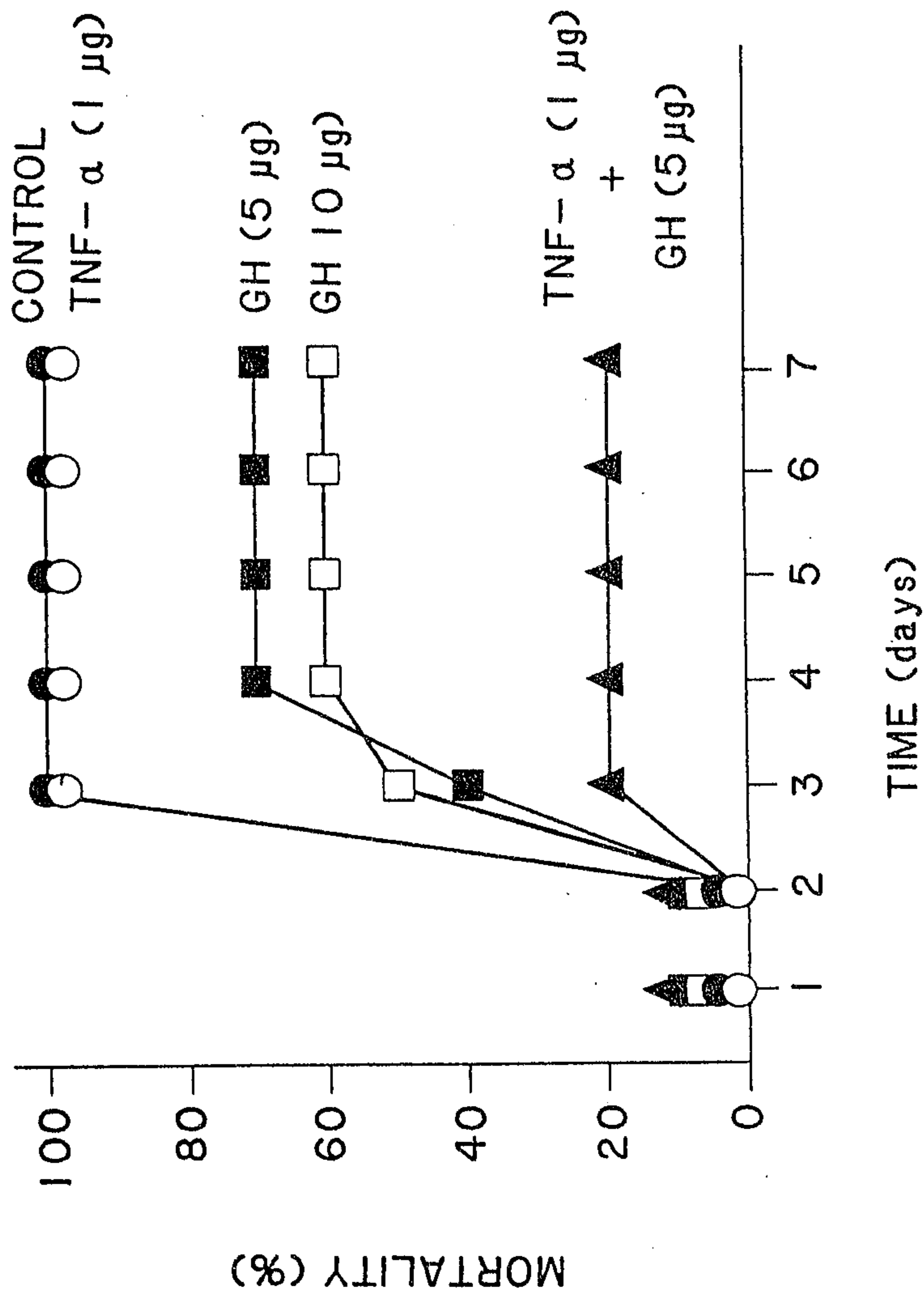


FIG. 5

5 / 5

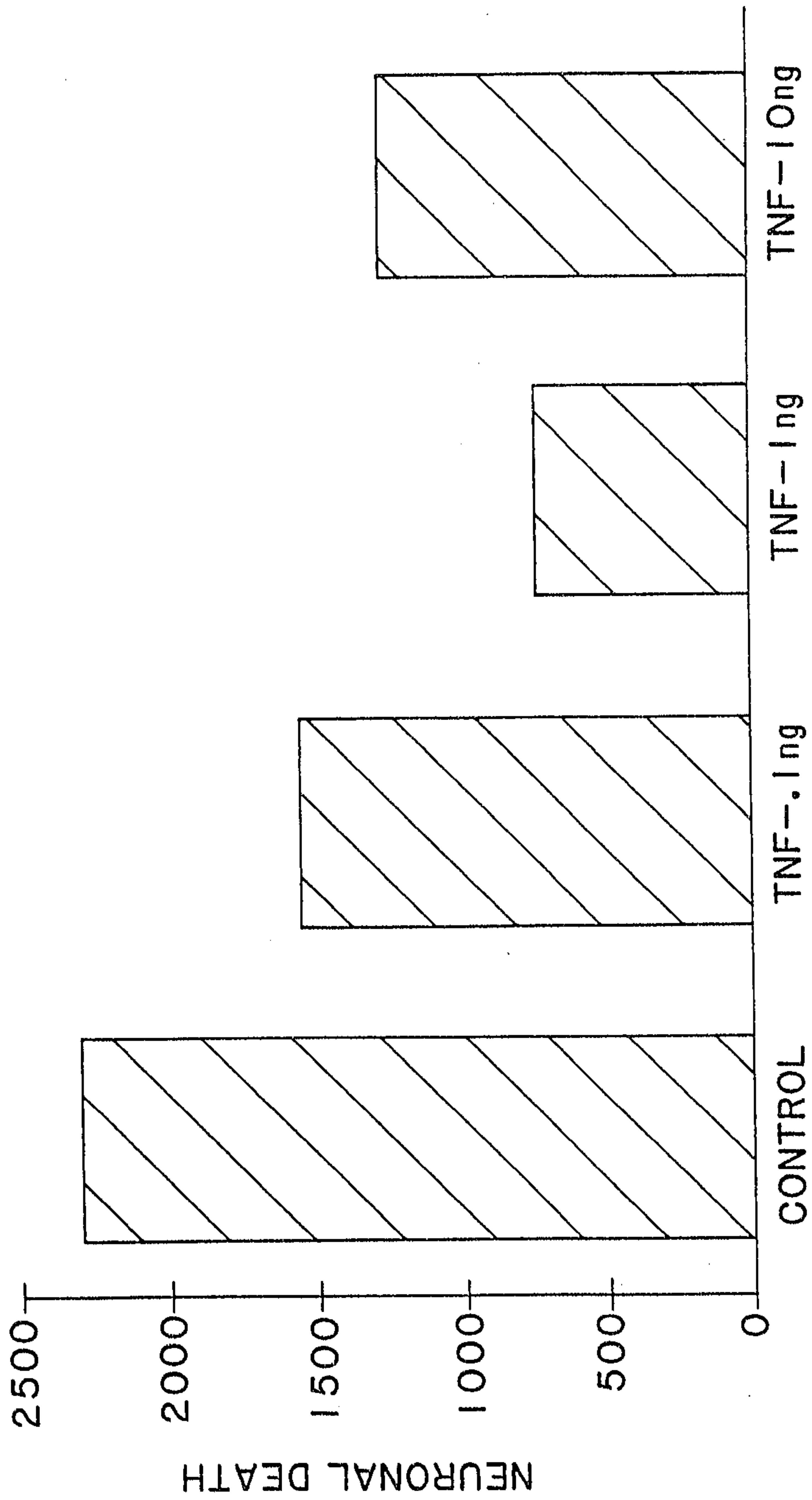


FIG. 6