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(71) Applicant *(for all designated States except US)*: **CENTER FOR BLOOD RESEARCH, INC.** [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **REMOLD-O'DONNELL, Eileen** [US/US]; 197 Clinton Road, Brookline, MA 02445 (US).

(74) Agents: **DENNIS, Emma, A.** et al.; 4400 Biscayne Boulevard, Miami, FL 33137 (US).

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(54) Title: MODULATORS OF ELASTASE INHIBITOR SECRETION

(57) Abstract: The invention provides methods and agents for stimulating the release of monocyte/neutrophil elastase inhibitor from a cell, and the invention also provides methods for identifying agents that stimulate monocyte/neutrophil elastase inhibitor release from a cell. The methods and agents disclosed are useful in the management of inflammatory conditions and diseases.

MODULATORS OF ELASTASE INHIBITOR SECRETION

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the fields of cell biology, biochemistry, medicine, and pharmacology.

Summary of the Related Art

The serpins (serine protease inhibitors) and related proteins constitute one of the earliest described protein superfamilies recognized by Hunt and Dayhoff in 1981 by computer analysis of amino acid sequence identity (Hunt *et al.* (1981) *Biochem. Biophys. Res. Commun.* 95:864-871). One group in this superfamily is the "Ov-familiy", which would not be recognized as such based on sequence identity alone (Remold-O'Donnell, E. (1993) *FEBS* 315:105-108).

Examples of Ov-family members include human squamous cell antigen, human plasminogen activator (PAI-2), human monocyte/neutrophil elastase inhibitor (MNEI), chicken ovalbumin (Oval), and chicken gene Y. (*id.*) Ov-family proteins lack the N-terminal extension region and a cleavable hydrophobic signal sequence, two properties normally associated with serpin family members. Several members of this family appear to rely on a non-cleavable internal signal sequence, since they exist as dualistic molecules that can be either secreted or located cytoplasmically.

Maintenance of the delicate balance of phagocytic cell proteases and protease inhibitors is critical for preservation of the integrity of local organ function. Loss of this balance may be a major causative factor in the pathogenesis of emphysema, asthma, chronic bronchitis, sarcoidosis, bronchiectasis, respiratory distress syndrome, arthritis and certain skin diseases.

Human monocyte/neutrophil elastase inhibitor (MNEI) (GenBank Accession No. P30740) is a serpin superfamily protein that rapidly inhibits the neutrophil granule proteases elastase (GenBank Accession No. AAD45239), cathepsin G (cat G) (GenBank Accession No. XP_007318), and proteinase-3 (PR-3) (GenBank Accession No. XP_009264). MNEI was first detected in secretions from cultured macrophages and is believed to play a central role in the control of extracellular serine protease activity in the lung. MNEI is found at elevated concentrations in the bronchoalveolar lavage (BAL) fluid of cystic fibrosis patients with severe lung inflammation, suggesting increased release in response to inflammatory conditions.

There are no known compounds and methods to stimulate the cellular secretion or release of MNEI. Such compounds and methodologies would be useful in the control of inflammatory conditions and diseases.

BRIEF SUMMARY OF THE INVENTION

It has surprisingly been discovered that MNEI is released from cells by way of a novel secretory pathway and that various agents are useful for stimulating the cellular secretion or release of MNEI. These discoveries have been utilized to provide the present invention, which includes methods for stimulating the release of MNEI from a cell and methods of identifying agents that stimulate MNEI release from a cell. These methods are useful in the treatment of inflammatory conditions and diseases.

In one aspect, the invention provides a method of stimulating the release of MNEI from a cell. The method comprises contacting the cell with an agent that stimulates the release of MNEI from the cell. In another embodiment the agent is a protease. In a certain embodiment, the protease is a neutrophil granule protease. In yet another certain embodiment, the protease is selected from the group consisting

of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin. In another certain embodiment, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. In other embodiments, the agent is a small molecule agonist of MNEI release.

In another aspect the invention provides a method of stimulating the secretion of MNEI from a cell. The method comprises contacting the cell with a priming agent followed by contacting the cell with an agent that stimulates the release of MNEI from the cell. In a certain embodiment the agent is a protease. In another certain embodiment, the protease is a neutrophil granule protease. In yet another embodiment, the protease is selected from the group consisting of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin. In other embodiments, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. In a certain embodiment, the priming agent is selected from the group consisting of lipopolysaccaride (LPA) or cyclohexamide (CHX). In other embodiments, the agent is a small molecule agonist of MNEI release.

In another aspect the invention provides a method of stimulating the secretion of MNEI from a cell. The method comprises contacting the cell with a priming agent and an agent that stimulates the release of MNEI from the cell. In a certain embodiment, the priming agent is etoposide (etop). In some embodiments, the agent that stimulates the release of MNEI from the cell is a protease. In a certain embodiment, the protease is a neutrophil granule protease. In another certain embodiment, the protease is selected from the group consisting of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin. In other embodiments, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or

chymotrypsin. In another certain embodiment, the agent is a small molecule agonist of MNEI release.

The invention provides a method of identifying an agent that stimulates the release of MNEI from a cell. The method comprises contacting the cell with a test agent and assaying for MNEI release, wherein an increase in the release of MNEI compared to a control sample not treated with the test agent indicates that the test agent stimulates the release of MNEI from the cell. In a certain embodiment, the test agent is a small molecule agonist of MNEI release.

In another aspect, the invention provides a method for the treatment of inflammation. The method comprises contacting a cell with an agent that stimulates the release of MNEI, thereby reducing inflammation. In a certain embodiment, the treatment of inflammation is directed to a patient with cystic fibrosis, emphysema, asthma, chronic bronchitis, sarcoidosis, bronchiectasis, respiratory distress syndrome, arthritis and certain skin diseases. In particularly preferred embodiments, the method is used for the treatment of inflammation in patients with cystic fibrosis.

In yet another aspect, the invention provides a pharmaceutical composition useful for the treatment of inflammatory conditions and diseases. The composition comprises an agent that stimulates the release of MNEI and an acceptable pharmaceutical carrier. In some embodiments, the agent that stimulates the release of MNEI from the cell is a protease. In a certain embodiment, the protease is a neutrophil granule protease. In another certain embodiment, the protease is selected from the group consisting of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin. In yet another certain embodiment, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. In other embodiments, the agent is a small molecule agonist of MNEI release.

In another aspect, the invention provides a kit comprising a pharmaceutical composition useful for the treatment of inflammatory conditions or diseases and printed instruction on the use of the composition for the treatment of inflammatory conditions or diseases. The pharmaceutical composition of the kit comprising an agent that stimulates the release of MNEI. In certain embodiments, this agent is a protease. In a certain embodiment, the protease is a neutrophil granule protease. In another certain embodiment, the protease is selected from the group consisting of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin. In yet another certain embodiment, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. In other embodiments, the agent is a small molecule agonist of MNEI release.

As used herein, the phrase "stimulates release of MNEI" refers to a property of an agent, e.g., a protein or chemical compound, that causes an increase in the amount of MNEI secreted from the cell. Alternatively, the term "stimulate" may refer to the production and release of MNEI *de novo*.

As used herein, the term "protease" refers to an endopeptidase that cleave proteins into small fragments. More specifically, the term refers to serine protease, a group of proteins which share a common reaction mechanism based on the formation of an acyl-enzyme intermediate on a specific active serine residue. Serine proteases are all irreversibly inactivated by a series of organophosphorus esters, such as di-isopropylfluorophosphate (DFP) and by naturally-occurring inhibitors (*e.g.*, serpins).

The word "cell" is meant to encompass one that normally expresses MNEI. Non-limiting examples include monocytes and neutrophils.

The word "small molecule agonist" as used herein refers to a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably

less than 600 daltons (Da), which is capable of stimulating the release of MNEI from a cell.

As used herein, the term "analog" refers to a compound having a related structure and at least a measurable amount of similar activity as the reference compound.

The term "neutrophil granule protease" is meant to encompass a protease normally occurring in neutrophil granules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a general schematic presentation of the method of stimulating MNEI release from a cell.

Figure 2A is a graphic representation showing the effect of different agents on MNEI secretion.

Figure 2B is a graphic representation showing the viability of cells utilized in experiments performed to determine the affect of different agents on MNEI secretion.

Figure 3A is a graphic representation showing the ability of human neutrophil elastase to stimulate the release of MNEI in a dose-dependent manner.

Figure 3B is a graphic representation showing the results of control experiments designed to determine the percent of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) released by cells in experiments designed to demonstrate that human neutrophil elastase stimulates the release of MNEI in a dose-dependent manner.

Figure 4 is a graphic representation showing that various neutrophil granule proteases stimulate the release of MNEI.

Figure 5A is a graphic representation showing that pretreatment with cyclohexamide (CHX) increases cathespsin G-induced stimulation of MNEI release.

Figure 5B is a graphic representation showing that pretreatment with lipopolysaccarhide (LPS) increases neutrophil elastase-induced stimulation of MNEI release.

Figure 5C is a graphic representation showing that co-incubation with etoposide (etop) increases neutrophil elastase-induced stimulation of MNEI release.

Figure 6 is a graphic representation showing the kenetics of elastase- and cathepsin G-induced MNEI release.

Figure 7B is a representation of a Western blot showing that the release of MNEI is not affected by treatment with bredfeldin A, and indicating that this protein is not released by the endoplasmic reticulum/Golgi pathway.

Figure 7B is a representation of a Western blot showing that the release of TNF-α is inhibited by treatment with bredfeldin A, indicating that this protein is released by the endoplasmic reticulum/Golgi pathway.

DETAILED DESCRIPTION OF THE INVENTION

The patent and scientific literature cited herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references cited herein are hereby incorporated by reference.

Aspects of the invention utilize techniques and methods common to the fields of molecular biology, cell biology and immunology. Useful laboratory references for these types of methodologies are readily available to those skilled in the art. See, for example, Molecular Cloning, A Laboratory Manual, 2nd. edition, edited by Sambrook, J., Fritsch, B. F. and Maniatis, T., (1989), Cold Spring Harbor Laboratory Press; Current Protocols In Molecular Biology and Current Protocols in Immunology, Wiley Interscience, New York; Harlow & Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1988).

The invention discloses a novel pathway for the release of MNEI from a cell. As will be apparent to those skilled in the art, the ability to stimulate the release of MNEI from a cell provides a tool for the management of inflammatory conditions and diseases. For example, the invention is directed, in part, to the management of cystic fibrosis by way of stimulating the release of MNEI from a cell.

In one aspect, the invention provides a method of stimulating the release of MNEI from a cell. The method comprises contacting the cell with an agent that stimulates the release of MNEI from the cell. Cells useful in the method of the invention include, but are not limited to, monocytes and neutrophils. Stimulation of the cells utilized in the methods of the invention may be done *in vitro* or *in vivo*. The isolation and handling of such cells being well within the skill of those in the art.

In a certain embodiment the agent is a protease. In a certain embodiment, the protease is a neutrophil granule protease. Alternatively, the protease is monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, chymotrypsin, an analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. The agent may also be a small molecule agonist of MNEI release.

In another aspect the invention provides a method of stimulating the secretion the secretion of MNEI from a cell. The method comprises contacting the cell with a priming agent followed by contacting the cell with an agent that stimulates the release of MNEI from the cell. The agent may be a protease such as a neutrophil granule protease. Alternatively, the protease is monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin, or an analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. The priming agent is preferably selected from the group consisting of lipopolysaccaride (LPA) or cyclohexamide (CHX), or a small molecule agonist of MNEI release.

In another aspect the invention provides a method of stimulating the secretion the secretion of MNEI from a cell. The method comprises contacting the cell with a priming agent and an agent that stimulates the release of MNEI from the cell. Preferably, the priming agent is etoposide (etop). Preferably, the agent that stimulates the release of MNEI from the cell is a protease, such as a neutrophil granule protease or monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin or analog thereof. Alternatively, the agent is a small molecule agonist of MNEI release.

In another aspect, the invention provides a method of identifying an agent that stimulates the release of MNEI from a cell. The method comprises contacting the cell with a test

agent and assaying for MNEI release, wherein an increase in the release of MNEI compared to a control sample not treated with the test agent indicates that the test agent stimulates the release of MNEI from the cell. Preferably, the test agent is a small molecule agonist of MNEI release.

The invention disclosed herein encompasses the use of different libraries for the identification of small molecule agonists of MNEI secretion. Libraries useful for the purposes of the invention include, but are not limited to, (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides and/or organic molecules.

Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" or "leads" via natural product screening. By way of non-limiting example, monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, and chymotrypsin are lead agents for the design of analogs that stimulate MNEI release. Thus, in another certain embodiment, the protease is a analog of monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, or chymotrypsin. Natural product libraries are derived from collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see, Cane, D.E., *et al.* (1998) *Science* 282:63-68.

Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries.

More specifically, a combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

For a review of combinatorial chemistry and libraries created therefrom, see Huc, I. and Nguyen, R. (2001) *Comb. Chem. High Throughput Screen* 4:53-74; Lepre, C.A. (2001) *Drug Discov. Today* 6:133-140; Peng, S.X. (2000) *Biomed. Chromatogr.* 14:430-441; Bohm, H.J. and Stahl, M. (2000) *Curr. Opin. Chem. Biol.* 4:283-286; Barnes, C. and Balasubramanian, S. (2000) *Curr. Opin. Chem. Biol.* 4:346-350; Lepre, Enjalbal, C., *et al.* (2000) *Mass Septrom Rev.* 19:139-161; Hall, D.G., (2000) *Nat. Biotechnol.* 18:262-262; Lazo, J.S., and Wipf, P. (2000) *J. Pharmacol. Exp. Ther.* 293:705-709; Houghten, R.A., (2000) *Ann. Rev. Pharmacol. Toxicol.* 40:273-282; Kobayashi, S. (2000) *Curr. Opin. Chem. Biol.* (2000) 4:338-345; Kopylov, A.M. and Spiridonova, V.A. (2000) *Mol. Biol. (Mosk)* 34:1097-1113; Weber, L. (2000) *Curr. Opin. Chem. Biol.* 4:295-302; Dolle, R.E. (2000) *J. Comb. Chem.* 2:383-433; Floyd, C.D., *et al.* (1999) *Prog. Med. Chem.* 36:91-168; Kundu, B., *et al.* (1999) *Prog. Drug Res.* 53:89-156; Cabilly, S. (1999) *Mol. Biotechnol.* 12:143-148; Lowe, G. (1999) *Nat. Prod. Rep.* 16:641-651; Dolle, R.E. and Nelson, K.H. (1999) *J. Comb. Chem.* 1:235-282; Czarnick, A.W. and Keene, J.D. (1998) *Curr. Biol.* 8:R705-R707; Dolle, R.E. (1998) *Mol. Divers.* 4:233-256; Myers, P.L., (1997) *Curr. Opin. Biotechnol.* 8:701-707; and Pluckthun, A. and Cortese, R. (1997) *Biol. Chem.* 378:443.

Devices for the preparation of combinatorial libraries are commercially available (see, *e.g.*, 357 MPS, 390 MPS, Advanced Chem-Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). In addition, numerous combinatorial libraries are themselves commercially available (see, *e.g.*, ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Mo., ChemStar, Ltd., Moscow, RU, 3D Pharmaceuticals, Exton, Pa., Martek Biosciences, Columbia, Md., etc.).

Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries.

Small molecule agonists of MNEI release are identified and isolated from the libraries described herein by any method known in the art, *e.g.*, functional screening assays. and affinity binding methodologies. By way of non-limiting example, experiments detailed herein provide a general assay for the determination of whether an agent stimulates MNEI release from a cell. In addition, the screening methods utilized for the identification of small molecule agonists of MNEI secretion include high throughput assays.

In another aspect, the invention provides a method for the treatment of inflammation. The method comprises contacting a cell with an agent that stimulates the release of MNEI, thereby reducing inflammation. Preferably, the treatment of inflammation is directed to a patient with cystic fibrosis, emphysema, asthma, chronic bronchitis, sarcoidosis, bronchiectasis, respiratory distress syndrome, arthritis and certain skin diseases. For example, the methods are useful for the treatment of inflammation in patients with cystic fibrosis.

In another aspect, the invention provides a kit comprising a pharmaceutical composition useful for the treatment of inflammatory conditions or diseases and printed instruction on the use of the composition for the treatment of

inflammatory conditions or diseases. The pharmaceutical composition of the kit comprising an agent that stimulates the release of MNEI. Preferably, the agent that stimulates the release of MNEI from the cell is a protease, such as a neutrophil granule protease, In monocyte/neutrophil elastase, cathepsin G, proteinase-3, pancreatic elastase, chymotrypsin, and analogs thereof. Preferably, the agent is a small molecule agonist of MNEI release.

Pharmaceutical compositions of the invention are readily prepared using methods known in the art. For example, Remington's Pharmaceutical Sciences 17th ed., (1990) Mack Publishing Company, Easton, PA; and Pharmaceutical Dosage Forms and Drug Delivery Systems, 6th ed., Williams & Wilkins (1995) provide guidance to the practitioner for the preparation of pharmaceutical compositions.

The agents useful according the invention are included in pharmaceutical compositions of the invention at about 0.0001% to about 50% by weight, or more preferably at about 0.001% to about 10% by weight, or most preferably at about 0.05% to about 5% by weight.

The invention having been described, the following examples directed to the use of such compositions to lighten skin are offered by way of illustration and not limitation.

EXAMPLES

Example 1

General Procedure for Stimulating MNEI Release

The general procedure for stimulating MNEI release is presented in Figure 1. Briefly, human monocyte-like THP-1 (ATCC NO. were obtained from the American Tissue Type Collection (ATCC) located in Manassas, VA. Cells were cultured in RPMI/5% endotoxin-free fetal calf serum (FCS) and incubated with various agents for the indicated times at 37°C in an incubator under 5% CO_2. When incubated with proteases, FCS was omitted. Where indicated, cells were preincubated or coincubated with a priming agent. Cells were harvested by centrifugation and cytosolic and nuclear fractions were prepared by lysis with 10mMTris/HCl, pH 7.6, 0.5%NP-40, 25mM KCl, 5mM MgCl2 0.5mg/ml EDTA, 50 µg/ml leupeptine, 2mM DFP (cytosol) and 10 mM Tris/HCl, pH8.0, 0.1% NP-40, 50 µg/ml leupeptine, 2mM DFP nuclear fractions).

The amount of MNEI in culture supernatant and cytosol was measured by ELISA and/or Western Blot. Unspecified release due to cell lysis was measured by determining the release of GAPDH (Western Blot) and by determination of cell viability by Trypan-Blue exclusion.

Apoptosis induced by various agents was monitored by degradation of PARP (Western Blot) in nuclear fractions and cleavage of pro-caspase-3 (Western blot) in the cytosolic fraction (data not shown).

Example 2

Influence of Various Reagents on MNEI Release

THP-1 cell culture and sample collection were done essentially as described in Example 1. THP-1 cells were incubated for 4 hours with MNEI or various agents known to affect non-classical secretion or to induce apoptosis. MNEI and GAPDH in culture supernatants were determined by ELISA and Western Blot, respectively. Cell viability was determined by Trypan Blue exclusion.

Results of the experiment are presented in Figures 2A and 2B(Cont = control; NE = neutrophil elastase (5 µg/ml); TNF = TNF-α (5 ng/ml); TBN = thrombin (0.3 U/ml); CHX = cycloheximide; MA - methylamine (10 mM); Col - colchicine (5 µg/ml); Glyb = glyburide (100 µM); PMA (10 nM); Etop = etoposide (25 µM); Staur = staurosporine (0.5 µM). Human neutrophil elastase (NE) stimulates MNEI release from THP-1 cells. Agents known to influence secretion of other proteins lacking N-terminal signal peptides do not affect MNEI secretion.

Example 3

Human Neutrophil Elastase (NE) Stimulates MNEI Release in THP-1 Cells in a Dose-Dependent Manner

THP-1 cell culture and sample collection were done essentially as described in Example 1. Briefly, THP-1 cells in serum-free medium were incubated with purified human neutrophil elastase (NE) at different concentrations for three hours. The amount of MNEI and GAPDH in culture supernatents was determined by Western blot as percent of total MNEI and GAPDH, respectively.

The results of the study are presented in Figures 3A and 3B and demonstrate that extracellular neutrophil elastase (NE) stimulates MNEI release in a dose-dependent manner.

Unspecific release, measured by release of the cytosolic protein GAPDH is not increased by elastase.

Example 4

Neutrophil Granule Serine Proteases Increase MNEI Release

THP-1 cell culture and sample collection were done essentially as described in Example 1. Briefly, THP-1 cells in serum-free medium were incubated for 4 hours with various proteases at 5 µg/ml. MNEI release into culture supernatants was monitored by Western Blot and is graphed as release relative to control cells without protease treatment.

The results of the experiment are presented in Figure 4 and demonstrate that incubation with the neutrophil proteases elastase (NE), cathepsin G (Cat G), proteinase 3 (PR-3), as well as with porcine pancreatic elastase (PPE) and chymotrypsin (CT) induces an increase in MNEI secretion. These proteases are inhibited by MNEI *in vitro* (data not shown). Incubation of THP-1 cells with DPF-inactivated neutrophil elastase does not increase MNEI secretion.

Example 5

"Priming" Enhances Protease-Induced MNEI Secretion

THP-1 cell culture and sample collection were done essentially as described in Example 1. Briefly, after 3 hours activation with 25 µM CHX or 1 µg/ml LPS respectively, THP-1 cells in serum-free medium were incubated with cathepsin G (10 µg/ml) or human neutrophil elastase (NE, 10 µg/ml) for 3 hours. Alternatively, cells were co-incubated with etoposide (25 µM) and NE (10 µg/ml) for 4 hours. Control cells were not pre-incubated and not treated with protease. The release of MNEI into the culture supernatants was measured by Western blot (CHX and LPS) and by ELISA (etoposide).

The results of the experiment are presented in Figure 5A, 5B and 5C and demonstrate that pre-incubation of THP-1 cells

with cycloheximide (CHX), LPS, or co-incubation with etoposide (etop) enhances the increase of MNEI secretion induced by elastase or cathepsin G.

Example 6

Kinetics of Elastase- and Cathepsin G-Induced MNEI Release in THP-1 Cells

THP-1 cell culture and sample collection were done essentially as described in Example 1. Briefly, after 3 hours activation with 1 μg/ml LPS, THP-1 cells in serum-free medium were incubated with human neutrophil elastase (NE, 1 μg/ml) or cathepsin 3 (1 μg/ml) for times indicated. Cells activated with LPS but without protease served as control. The release of MNEI into the culture supernatants was monitored by Western blot.

The results of the experiment are presented in Figure 6 and demonstrate that Time course of MNEI secretion in activated THP-1 cells incubated with elastase or cathepsin G LPS alone also increased MNEI release as indicated by control samples without LPS pre-incubation. MNEI release during primary with LPS is indicated (dotted line). Proteases were added at time = 0.

Example 7

MNEI is Not Released Via the Endoplasmic Reticulum (ER)/ Golgi Pathway

THP-1 cell culture and sample collection were done essentially as described in Example 1. Briefly, after 3 hours pre-incubation with 1 μg/ml LPS, THP-1 cells in serum-free medium were incubated for 3 hours with purified human neutrophil elastase (NE) at 10 μg/ml or brefeldin A (0.5 μg/ml). The release of MNEI and TNF-α into the culture supernatants was monitored by Western blot.

The results of the experiment are presented in Figure 7A and 7B and demonstrate that MNEI secretion is not inhibited by brefeldin A, a fungal metabolite that blocks protein translocation from the ER to the Golgi. Therefore, MNEI secretion does not occur via the classical ER/Golgi pathway. In contrast to MNEI, secretion of TNF-α, a protein secreted via the ER/Golgi pathway, is completely blocked by brefeldin A.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed:

1. A method for stimulating the release of a monocyte/neutrophil elastase inhibitor from a human cell comprising contacting the human cell with a neutrophil elastase, wherein the contacted human cell releases monocyte/neutrophil elastase inhibitor.

2. The method of claim 1, wherein the neutrophil elastase is from a human.

3. The method of claim 1, wherein the human cell contacted with the neutrophil elastase releases the monocyte/nutrophil elastase in a dose-dependent manner.

4. The method of claim 1, wherein the human cell is in a patient suffering from inflammation.

5. The method of claim 4, wherein the patient is suffering from an inflammatory disease selected from the group consisting of cystic fibrosis, emphysema, asthma chronic bronchitis, sarcoidosis, brochiectasis, respiratory distress syndrome, arthritis, and a skin disease.

6. The method of claim 1, wherein the release of the monocyte/neutrophil elastase inhibitor from the contacted cell is not blocked by an agent that blocks protein translocation via a classical endoplasmic reticulum/Golgi apparatus pathway.

7. The method of claim 6, wherein the agent is brefeldin A.

8. A method for stimulating the release of monocyte/neutrophil elastase inhibitor from a human cell comprising contacting the human cell with a protease, the protease is being selected form the group consisting of neutrophil elastase, cathepsin G, proteinase-3, porcine pancreatic elastase, and chymotrypsin, wherein the contacted human cell releases monocyte/neutrophil elastase inhibitor.

9. The method of claim 8, wherein the neutrophil elastase is from a human.

10. The method of claim 8, wherein the human cell is in a patient suffering from inflammation.

11. The method of claim 10, wherein the patient is suffering from an inflammatory disease selected from the group consisting of cystic fibrosis, emphysema, asthma chronic bronchitis, sarcoidosis, brochiectasis, respiratory distress syndrome, arthritis, and a skin disease.

12. A method for stimulating the release of monocyte/neutrophil elastase inhibitor from a human cell comprising contacting the human cell with a first agent, the first agent being selected from the group consisting of cyclohexamide, lipopolysaccharide, and etoposide; and contacting the human cell with a second agent, the second agent being selected from the group consisting of cathepsin G and neutrophil elastase,

wherein the human cell contacted with the first agent and the second agent releases monocyte/neutrophil elastase inhibitor.

13. The method of claim 12, wherein the neutrophil elastase is from a human.

14. The method of claim 12, wherein the human cell is in a patient suffering from inflammation.

15. The method of claim 12, wherein the patient is suffering from an inflammatory disease selected from the group consisting of cystic fibrosis, emphysema, asthma chronic bronchitis, sarcoidosis, brochiectasis, respiratory distress syndrome, arthritis, and a skin disease.

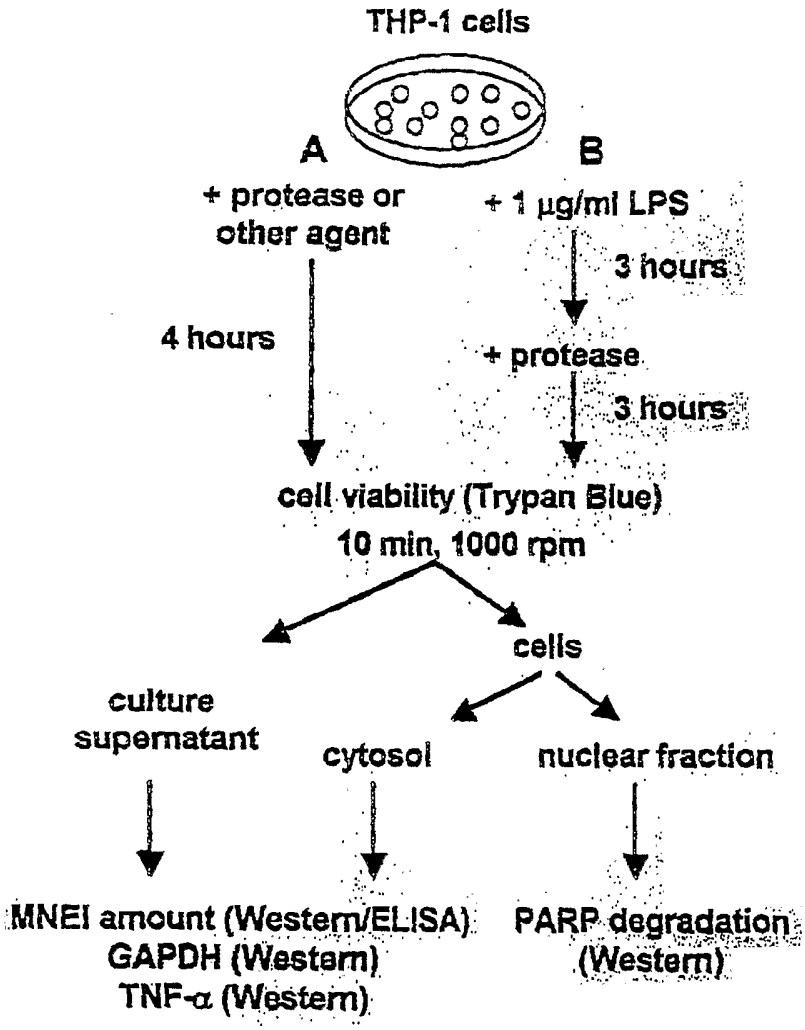
THP-1 cells
A
B
+ protease or other agent
+ 1 µg/ml LPS
3 hours
4 hours
+ protease
3 hours
cell viability (Trypan Blue)
10 min, 1000 rpm
cells
culture supernatant
cytosol
nuclear fraction
MNEI amount (Western/ELISA)
GAPDH (Western)
TNF-α (Western)
PARP degradation (Western)

Figure 1

Influence of different agents on MNEI release

specific MNEI release (ratio MNEI release: GAPDH release)

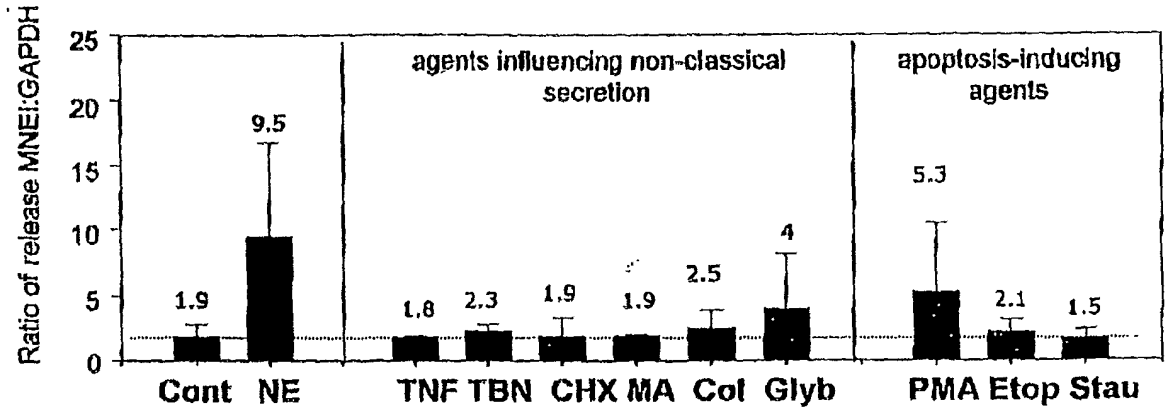

Figure 2A

cell viability

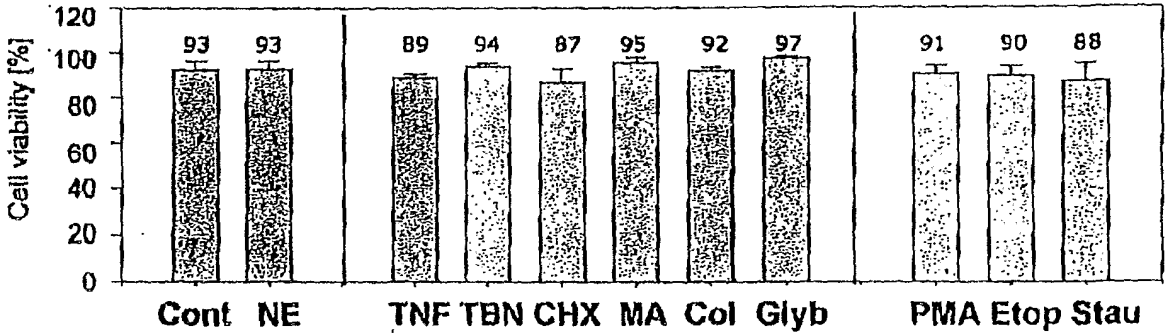

Figure 2B

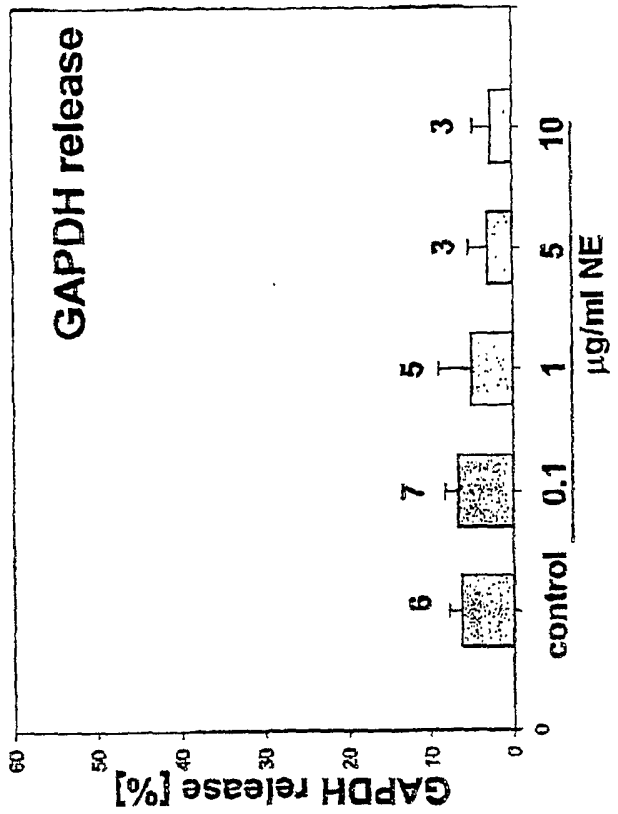

Figure 3B

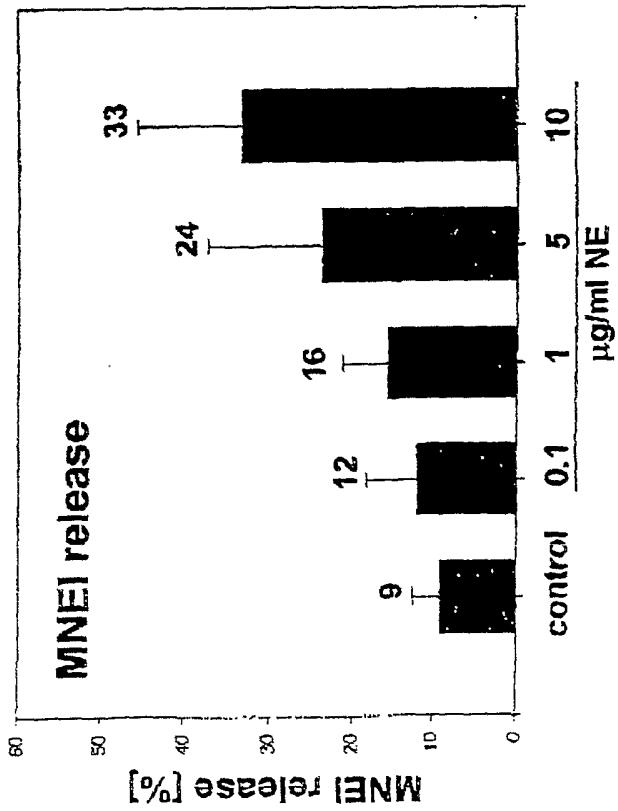

Figure 3A

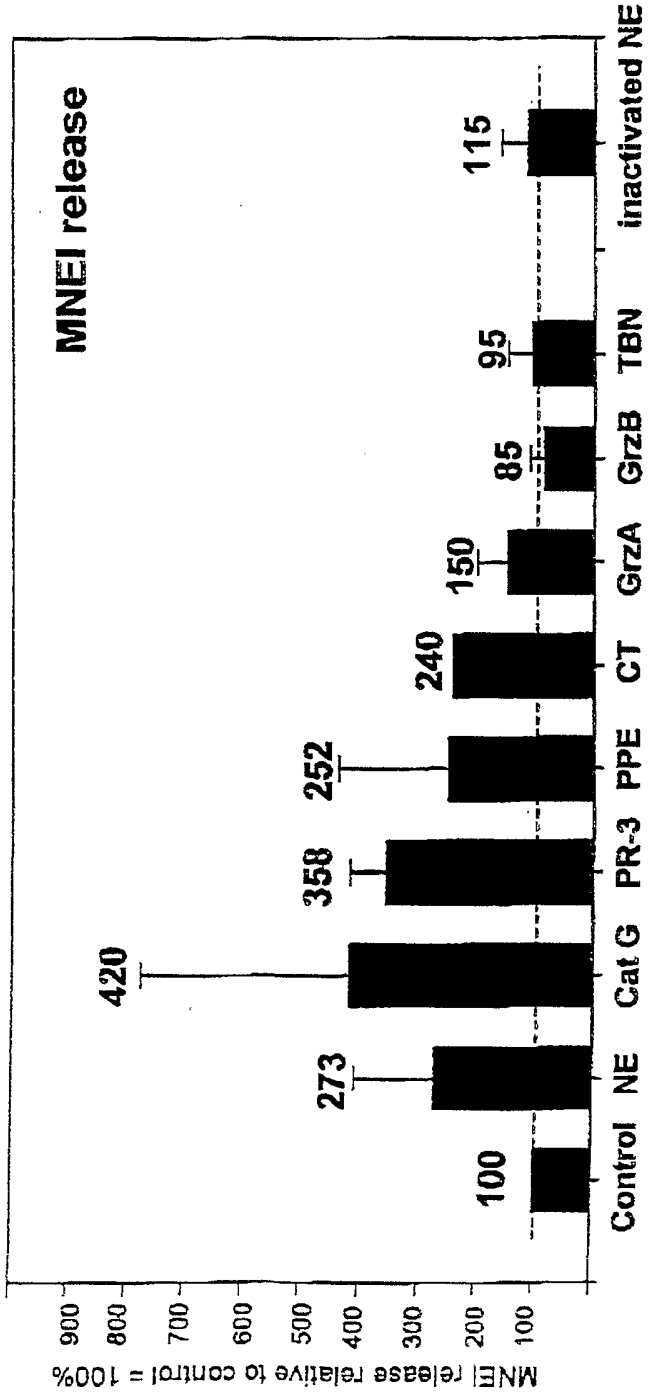
MNEI release
MNEI release relative to control = 100%
100
273
420
358
252
240
150
85
95
115
Control
NE
Cat G
PR-3
PPE
CT
GrzA
GrzB
TBN
inactivated NE

Figure 4

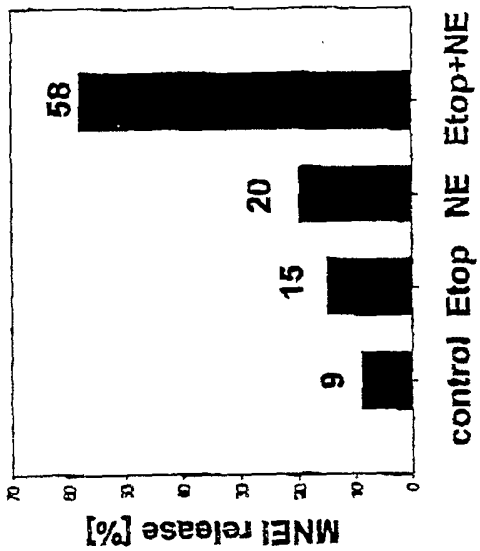

Figure 5C

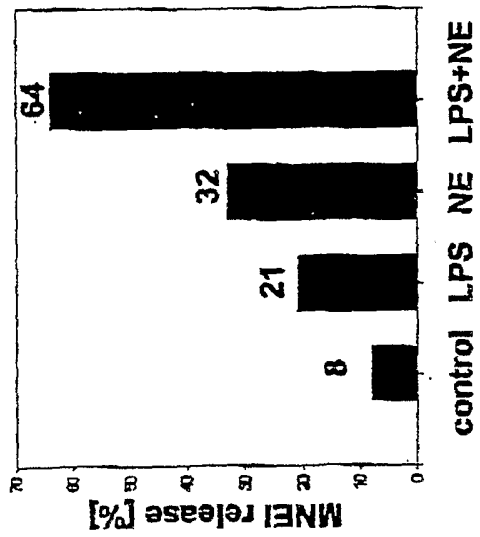

Figure 5B

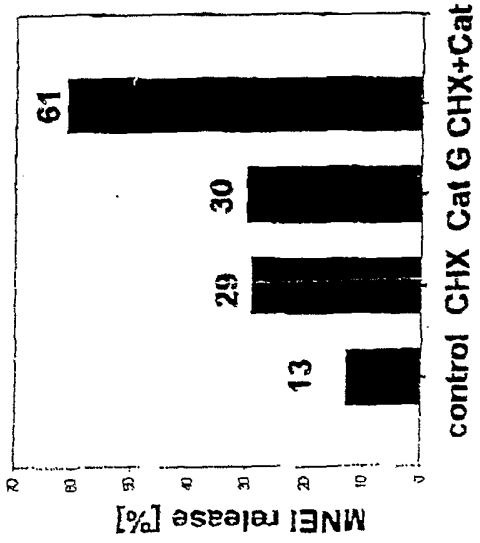

Figure 5A

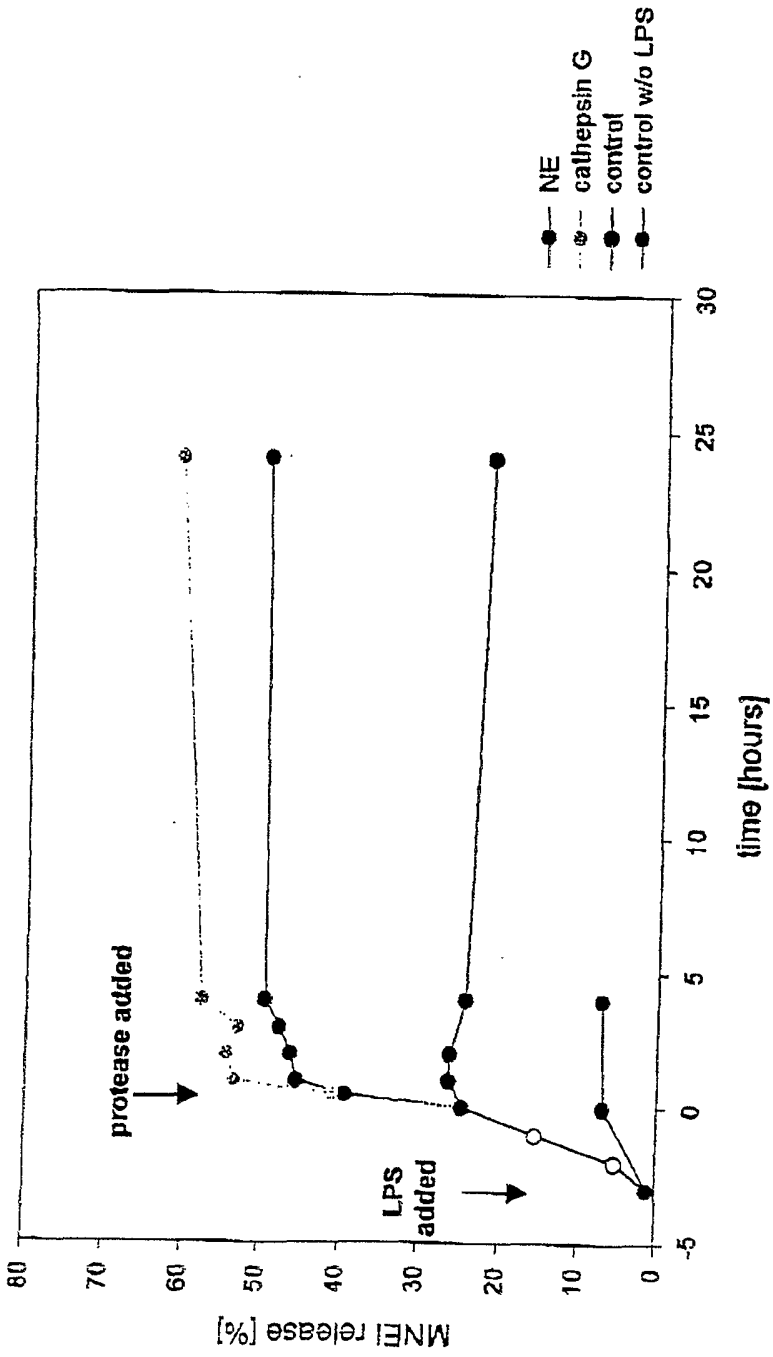
NE
cathepsin G
control
control w/o LPS
protease added
LPS added
MNEI release [%]
time [hours]
0
10
20
30
40
50
60
70
80
-5
0
5
10
15
20
25
30

Figure 6

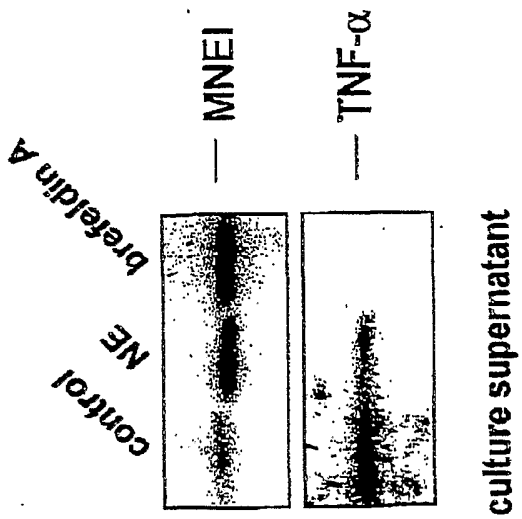

Figure 7A

Figure 7B