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(54) Title: INFLAMMATORY ACTIVITY DETECTION METHOD USING IL-6

(57) Abstract: Inflammatory activity contaminating a medical device or biologic agent can greatly affect both its safety and its efficacy. Disclosed are methods of detecting inflammatory activity in a test sample by administering the test sample to an animal and detecting the level of a pro-inflammatory cytokine or other cell-derived mediator of inflammation. Elevated levels of pro-inflammatory cytokines such as IL-1, IL-6, TNF- $\alpha$ , or IFN- $\gamma$ , or of other cell-derived mediators of inflammation such as lysosome granules, histamine, IL-8, leukotriene B4, nitric oxide, or prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), indicate that the test sample has inflammatory activity. The inflammatory activity may be due to any pro-inflammatory factor, such as a bacterial endotoxin (e.g., a lipopolysaccharide) or a non-endotoxin pyrogen (e.g., a *Streptococcal* mucopeptide).



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## INFLAMMATORY ACTIVITY DETECTION METHOD USING IL-6

### Background of the Invention

[001] When certain chemical or biological compounds are brought into contact with the circulatory system of humans or other mammals, they can cause a systemic inflammatory response. The inflammatory response is a defense mechanism to protect the body from infection and/or injury; inflammation increases blood flow to the site of infection or injury, bringing necessary fluids, proteins, and white blood cells (leukocytes) to help in the healing process. For example, one symptom associated with the inflammatory response is an elevation in body temperature or fever, which functions as a defense mechanism to pathogens that cause overheating. The inflammatory response is often, therefore, associated with a variety of "flu-like" symptoms including fever, chills, fatigue, headaches, loss of appetite, and muscle stiffness. A chemical or biological compound that triggers fever has historically been referred to as a "pyrogen" or a "pyrogenic" compound, referring to the fever response which such compounds may cause. Some chemical or biological compounds, however, are generally pro-inflammatory and may or may not cause fever as part of the inflammatory response that they cause.

[002] In some cases, depending on the sensitivity of an individual and the type and concentration of inflammatory agent the individual is exposed to, an individual can develop life-threatening shock-like symptoms after exposure to an inflammatory agent. Medical products which can be inhaled, injected, or infused and medical devices such as membranes or implanted materials pose a particular risk of pyrogenicity. Even nutrients can represent a risk of pyrogenicity. Inflammatory agents, or pyrogens, contained in medical products and nutrients are referred to as exogenous inflammatory agents (or pyrogens); in contrast, endogenous pyrogens are messenger compounds of the immune system that mediate an individual's inflammatory response to exogenous pyrogens. In addition to the pyrogenic nature of a product itself or by-products of its production, contamination of the product can often cause pyrogenicity.

[003] Pyrogenicity due to contamination of a product can be caused by any one of a diverse group of pyrogens derived from bacteria, viruses, fungi, or even from the host. This problem can persist even if the product is "sterilized" by heat or chemical methods. A commonly encountered pyrogenic compound, bacterial endotoxin (consisting largely of lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria), can remain after the bacteria are killed. In addition, Gram-positive bacteria, such as *Streptococcus*, produce pro-inflammatory cell wall mucopeptides. Thus, pyrogen testing of various pharmaceuticals, nutrients, and medical products for parenteral application is necessary in order to ensure the safety of such products.

[004] Indeed, immune and/or inflammatory responses to implanted medical devices, as well as non-vaccine biologics, can affect their safety and efficacy, resulting in adverse events including administration reactions, hypersensitivity, deficiency syndromes and lack of clinical response. Accordingly, immunogenicity and inflammatory testing has become a key component in the demonstration of clinical safety and efficacy. In fact, it is highly unlikely that regulatory approval would be granted for an implanted medical device or a non-vaccine biologic without an assessment of these parameters (see Shankar, *et al.* (2006) Trends in Biotechnology 24:274-80). Indeed, the Food and Drug Administration (FDA) has published guidelines for the evaluation of the potential immunotoxicity of investigational new drugs (see "Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs" U.S. Department of Health and Human Services Food FDA Center for Drug Evaluation and Research (CDER), October 2002, available at [www.fda.gov/cder/guidance/index.htm](http://www.fda.gov/cder/guidance/index.htm)). These guidelines advise appropriate examination of new drug products for "adverse immunostimulation", including chronic inflammation, but that "[n]o general method is available for assessing the potential for a drug to produce adverse immunostimulation".

[005] The rabbit pyrogen test has been historically used to monitor pyrogen contamination in mass-produced pharmaceutical products. The rabbit test is an *in-vivo* test which consists of injecting a statistically significant number of rabbits with the sample compound intravenously and observing the average rise in

body temperature elicited in the test animals. Although the rabbit test is responsive to a wide spectrum of pyrogenic agents, including non-endotoxin pyrogens, the rabbit test has a relatively low sensitivity (ng endotoxin/ml). The relative insensitivity, poor quantitative and variability of results, as well as ethical issues involved in animal testing have made the rabbit test disfavored in recent years.

[006] Another inflammatory assay that has been developed is the rabbit ocular toxicity test (ROTT). In this assay, a portion of the natural rabbit eye vitreous fluid is removed and the volume is replaced with the same volume of HA sample. Two days after instillation, the vitreous fluid is sampled and the number of white blood cells present are counted. Crudely purified HA preparations produced much higher white blood cell counts (*i.e.* ROTT scores of ~800 cells/mm<sup>3</sup>) than did highly purified preparations of HA (*i.e.*, ROTT scores of < ~25 cells/mm<sup>3</sup>).

[007] The intravitreal injection of owl monkeys (*Aotus trivigatus*) has also been used to measure inflammatory activity, most frequently for preparation of bacterially synthesized hyaluronic acid. The owl monkey was used because it was found that, when fully grown, this new world monkey had a liquid vitreous (see Balazs *et al.* (1959) Comparative Biochemistry. Arch. Biochem Biophys. 81:464-79). The presence of a liquid rather than a gel vitreous was a prerequisite for these tests. The exchange of viscous liquid vitreous with the biopolymer solution had to be achieved with minimal surgical trauma in order to evaluate the tolerance of the vitreous substance used. The owl monkey vitreous test was consequently developed as an extremely sensitive method to evaluate the inflammatory reaction in the vitreous after the injection of viscoelastic solutions (see Balazs *et al.* (1966) "Replacement of the vitreous body of monkeys with reconstituted vitreous and hyaluronic acid" in Modern Problems in Ophthalmology (Surgery of retinal vascular diseases and prophylactic treatment of retinal detachment), Amersfoort 1963 (ed. Streiff, E. B.). Vol 4 pp. 230-32). The liquid vitreous of the owl monkey can be easily replaced without mechanical damage to the retina and lens. The minimal operational trauma permits a very accurate and sensitive evaluation of the tolerance of the vitreal implant. U.S. Pat. No.

4,141,973 describes a modified owl monkey test to test the inflammatory reaction of ultrapure hyaluronic acid.

[008] Another primate that has been used to test the inflammatory reaction of ultrapure hyaluronic acid is the rhesus monkey. Although the total volume of the vitreous of the adult rhesus eye is approximately 24% larger than the owl monkey eye, the volume of the liquid vitreous is smaller (0.8 to 1.2 ml) in the rhesus than the owl monkey eye (1.8-2.2 ml). In the rhesus monkey, only 0.5 ml of vitreous can be removed and replaced. By reducing the volume of vitreous removed and replaced, the incidence of eyeball collapse is greatly reduced, and therefore, there is less traumatic injury to the blood aqueous barrier. The rhesus monkey has been shown to have similar inflammatory reactions as the owl monkey, after vitreal injection of HEALON™. The rhesus monkey can also be used as an extremely sensitive system for detecting small amounts of inflammatory agents (see Denlinger and Balazs, (1980) "Replacement of the Liquid Vitreous with Sodium Hyaluronate in Monkeys" Experimental Eye Research 31:81-99.)

[009] The oxidative burst inflammation test is yet another method of testing for inflammatory activity. The oxidative burst (OB) inflammation test is a standard test for pyrogenic activity in which the activation of an acute phase immune response is measured in an animal (*e.g.*, mouse) after injection of a test sample by detecting transformation of molecular oxygen into reactive oxygen species (ROS) by leukocytes. The OB method is carried out by measuring the OB of cells harvested from the peritoneum of test sample-injected mice by means of UV spectrophotometric determination of cytochrome C oxidation in the peritoneal exudate cells (*i.e.*, mainly polymorphonuclear leukocytes (PMNs), which are the primary responders to inflammatory material). In PMNs, the oxidative burst may be amplified by the addition of phorbol myristate acetate (PMA). The cell-dependent OB response may then be determined by measuring the absorbance of freshly added cytochrome C at 550 nm. Under standard test conditions a non-inflammatory negative control will generally produce an absorbance of cytochrome C at 550 nm ( $A_{550}$ ) of  $\leq 0.10$  at  $1 \times 10^5$  cells/ml, while a sample having inflammatory activity will typically produce an  $A_{550}$  of 0.15-0.40 at  $1 \times 10^5$  cells/ml.

[010] While the rabbit ocular, owl monkey, rhesus monkey, OB and other tests are accepted methods for detecting inflammatory activity, they suffer from several inconveniences and inefficiencies. In particular, the simian assays are time-consuming, costly and of arguable value in view of ethical considerations. Furthermore, the OB test method has a relatively narrow signal-to-noise ratio, thereby giving rise to a large number of false positive and false negatives and necessitating repeated testing. Still further, the OB testing method frequently yields hemolytic samples obtained from animal handling that must be excluded from the test. In addition, the OB test for inflammatory activity relies upon a very limited sampling of immune leukocyte cells withdrawn from each individual mouse peritoneum, thereby increasing the potential for testing error. While the limited number of peritoneal cells from individual animals can be pooled, this technique impacts on the statistical analysis of the results and can yield a false positive result in cases where the number of cells from one animal are the majority that are tested for that group.

[011] Accordingly, additional, more accurate, efficient and reliable methods for detecting the inflammatory activity of a test sample would be useful.

### **Summary of the Invention**

[012] The invention is based, in part, upon the discovery that detection of interleukin-6 (IL-6), or another pro-inflammatory cytokine or other cell-derived mediator of inflammation, provides a particularly sensitive, accurate and convenient method for detecting inflammatory activity as compared to methods based upon detection of the oxidative burst (OB) produced by activated polymorphonuclear leukocytes. The elevation of IL-6 levels, or other cell-derived mediators of inflammation, in the peritoneal fluid of a test animal may be detected using any available method, such as an IL-6 specific ELISA (Enzyme-Linked ImmunoSorbent Assay). The method of the invention provides the additional advantage of not requiring the isolation of intact immune cells (*e.g.*, from peritoneal exudate), thereby allowing the use of hemolytic samples that are frequently obtained upon animal handling and which would otherwise have to be excluded when employing an oxidative burst assay. The method of the invention

further avoids the additional laborious testing of isolated cells in vitro, and produces more statistically reliable measurements.

[013] In one aspect, the invention provides a method of detecting inflammatory activity in a sample by administering a test sample to an animal, and detecting a level of interleukin-6 (IL-6) in the animal. By this method, an elevated level of IL-6 in the test animal, as compared to a control animal injected with a non-inflammatory control sample, indicates that the test sample has inflammatory activity.

[014] In particular embodiments of this method, the test sample may be a biological sample, a medical device or a biopolymer. Exemplary test samples for use in the method of the invention include heteropolysaccharide preparations such as hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin sulfate, heparin, heparan sulfate, agar, alginate, carrageenan, gellan, guar gum, locust bean gum and/or xanthan gum. In particularly useful embodiments the test sample is a hyaluronan (or hyaluronic acid or hyaluronate) preparation, e.g., an HA preparation for use in synovial fluid supplementation or as a dermatological filler agent.

[015] In certain embodiments, the heteropolysaccharide is produced by bacterial fermentation. In some embodiments, the heteropolysaccharide is produced by bacterium of the genus *Streptococcus*, and the inflammatory activity detected may be, e.g., a *Streptococcal* mucopeptide. In other embodiments, the inflammatory activity detected is a bacterial endotoxin, e.g., a lipopolysaccharide. In particular embodiments, the inflammatory activity detected by the method of the invention is from a Gram-positive bacterium, e.g., a lipoteichoic acid (LTA).

[016] The test animal used in the method may be, in particular embodiments, a mammal, e.g., a mouse, guinea pig, or rat. In certain embodiments, the test sample is administered by injecting it into the abdomen of the mammalian test animal.

[017] The level of IL-6 may be detected using any method or technique known in the art. For example, the level of IL-6 in the test animal may be detected using an IL-6 antibody; e.g., using an antibody-antigen-antibody sandwich

enzyme-linked immunosorbent assay (ELISA). Alternatively, the level of IL-6 may be detected with a bioassay that is sensitive to IL-6. In particular embodiments, the level of IL-6 in the animal is determined about 4-16 hours after administering the test sample to the animal.

[018] In another aspect the invention provides a method of detecting an exogenous non-endotoxin pyrogen in a Gram-positive bacterial fermentation product by injecting a mouse with the Gram-positive bacterial fermentation product intra-peritoneally; collecting a peritoneal fluid from the injected mouse at least about 6 hours after injecting the bacterial fermentation product; and measuring the IL-6 concentration in a cell free supernatant of the peritoneal fluid. By this method, an exogenous non-endotoxin pyrogen is present in the Gram-positive bacterial fermentation product if the IL-6 concentration is higher than the concentration of IL-6 in a control mouse injected with a non-inflammatory negative control.

[019] In particular embodiments, the exogenous non-endotoxin pyrogen is a *Streptococcal* mucopeptide. In some embodiments, the Gram-positive bacterial fermentation product is hyaluronic acid (HA). In certain embodiments, the Gram-positive bacterium is a *Streptococcus*; e.g., a hemolysin mutant strain of *Streptococcus zooepidemicus* such as HA-116 (ATCC 39920).

[020] In further embodiments, the mouse tested in the method of the invention is a Balb/c mouse, a CB6F1 mouse, or the progeny or descendent of a cross between a Balb/c and a CB6F1 mouse. In particular embodiments, the peritoneal fluid is collected from the mouse approximately 4-16 hours after injecting the Gram-positive bacterial fermentation product. The level of IL-6 may be detected using any method or technique known in the art. For example, the level of IL-6 in the test animal may be detected using an IL-6 antibody; e.g., using an antibody-antigen-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Alternatively, the level of IL-6 may be detected using a bioassay that is sensitive to IL-6. In particular embodiments of the method of the invention, the presence of an exogenous non-endotoxin pyrogen in the bacterial fermentation product is indicated if there is a statistically significant higher concentration of IL-6 concentration in the mouse injected with the Gram-positive bacterial fermentation

product than in the control mouse injected with the non-inflammatory negative control. In still further embodiments, the Gram-positive bacterial fermentation product is an otherwise non-immunogenic preparation of a heteropolysaccharide, *e.g.*, sodium hyaluronate.

[021] In yet another aspect, the invention provides a method of detecting inflammatory activity in a test sample by administering the test sample to an animal; and detecting a level of a pro-inflammatory cytokine or other cell-derived mediator of inflammation in the animal. By this method, an elevated level of the pro-inflammatory cytokine or other cell-derived mediator of inflammation in the test animal, as compared to a control animal injected with the non-inflammatory control sample, indicates that the test sample has inflammatory activity. In particular embodiments, the level of one or more particular pro-inflammatory cytokines, such as IL-1, IL-6, TNF- $\alpha$ , and/or IFN- $\gamma$ , is detected. In further embodiments, the level of one or more particular cell-derived mediators of inflammation, such as lysosome granules, histamine, IL-8, leukotriene B<sub>4</sub>, nitric oxide, and/or prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), is detected. In still further embodiments, the test sample is an otherwise non-immunogenic preparation of a heteropolysaccharide, *e.g.*, sodium hyaluronate.

### **Brief Description of the Figures**

[022] Figure 1 is a representation of potentially inflammatory bacterial membrane and cell wall components that may contaminate bacterial fermentation preparations including those occurring in Gram-positive bacteria (such as *Streptococcus*), Gram-negative bacteria, Mycoplasma and Mycobacterium tuberculosis.

[023] Figure 2 is a diagrammatic depiction of the cytochrome c assay for inflammatory activity in a test sample.

[024] Figure 3 is a diagrammatic depiction of the IL-6 ELISA assay for inflammatory activity in a test sample.

### **Detailed Description of the Invention**

#### General

[025] In general, the invention provides materials and methods for the detection of an inflammatory activity in a test sample, *e.g.*, any biomaterial. Despite the fact that most biomaterials are inert, nontoxic and nonimmunogenic, biomaterial implants can nonetheless often cause adverse reactions, particularly if they are contaminated by even minute amounts of an inflammatory activity (*e.g.*, a bacterial endotoxin, such as LPS, or cell wall mucopeptide, or other gram-positive bacterial inflammatory activity, *e.g.*, lipoteichoic acid).

[026] In addition, a particularly useful and normally non-immunogenic and non-inflammatory biomaterial, hyaluronan, has been shown to possess inflammatory activity when it is degraded down into low molecular weight fragments (see Gallo *et al.* (2004) J. Biol. Chem. 279:17079-84). Only small HA fragments of tetra- and hexasaccharide size (sHA), but not intermediate size (*e.g.*, M.W. 80 - 200 kD) or high molecular weight (*e.g.*, 600 - 1,000 kD) were shown to possess such inflammatory activity (Simon *et al.* (2000) J. Immunol. 165:1863-70). Accordingly the invention also provides a useful means of detecting inflammatory preparations of otherwise pure hyaluronan containing very low molecular weight hyaluronan fragments.

[027] Typically, shortly after implantation biomaterials may attract a layer of phagocytic cells (especially, macrophages, monocytes and neutrophils) and fibroblast-like cells. This process resembles the acute inflammatory response seen with many other types of foreign bodies and, to some extent, with focal bacterial infections. The acute inflammatory response to tissue contact biomaterials is very often followed by chronic inflammation and the appearance of fibrotic tissue surrounding many types of implants. Chronic inflammation and fibrosis are also associated with the degradation and failure of many types of biomaterial implants, including temporomandibular and other joint implants. Such inflammatory responses to inert, nonimmunogenic and nontoxic implanted biomaterials must be minimized. Accordingly, methods of detecting inflammatory activity in a biomaterial preparation are important to guarding against adverse reactions to biomaterials and other implanted matter.

### Definitions

[028] As used herein, the term "animal" is meant to include any eukaryotic metazoan exhibiting an inflammatory response to a foreign agent or tissue damage. Accordingly, the term "animal" includes all mammals such as mice, rats, guinea pigs, rabbits, humans, sheep, elks, deer, mule deer, minks, monkeys, horses, cattle, pigs, goats, dogs and cats. Also included within the scope of the term animal are birds, including chickens, as well as reptiles, fish, insects and arachnids.

[029] As used herein the term "antibody" refers to intact antibodies including polyclonal antibodies (see, for example *Antibodies: A Laboratory Manual*, Harlow and Lane (eds), Cold Spring Harbor Press, (1988)), and monoclonal antibodies (see, for example, U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and U.S. Pat. No. 4,411,993, and *Monoclonal Antibodies: A New Dimension in Biological Analysis*, Plenum Press, Kennett, McKearn and Bechtol (eds.) (1980)). The term "antibody" also refers to a fragment of an antibody such as F(ab), F(ab'), F(ab')<sub>2</sub>, Fv, Fc, and single chain antibodies which are produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. The term "antibody" also refers to bispecific or bifunctional antibodies, which are an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. (See Songsivilai *et al.*, Clin. Exp. Immunol. 79:315-321 (1990), Kostelny *et al.*, J. Immunol. 148:1547-1553 (1992)). As used herein the term "antibody" also refers to chimeric antibodies, that is, antibodies having a human constant antibody immunoglobulin domain is coupled to one or more non-human variable antibody immunoglobulin domain, or fragments thereof (see, for example, U.S. Pat. No. 5,595,898 and U.S. Pat. No. 5,693,493). Antibodies also refers to "humanized" antibodies (see, for example, U.S. Pat. No. 4,816,567 and WO 94/10332), minibodies (WO 94/09817), and antibodies produced by transgenic animals, in which a transgenic animal containing a proportion of the human antibody producing genes but deficient in the production of endogenous antibodies are capable of producing human antibodies (see, for example, Mendez *et al.*, Nature

Genetics 15:146-156 (1997), and U.S. Pat. No. 6,300,129). The term "antibodies" also includes multimeric antibodies.

[030] As used herein, the term "cytokine" refers to secreted proteins that are involved in regulation of the immune response including interleukins (ILs), interferons (IFNs), chemokines, tumor necrosis factors (TNFs), and a variety of colony stimulating factors (CSFs). Specific cytokines that may be measured in the assays of the invention include, but are not limited to, cytokines linked to TH1 response, cytokines linked to TH2 response, pro-inflammatory cytokines and/or cytokines such as IL-6, IL-1 $\beta$ , IL-12p70, IL-10, IL-2, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF- $\alpha$ , IL-8, IL-4, IL-5, IL-6, Eotaxin, IFN- $\alpha$ , IFN- $\gamma$ , soluble IL-6 receptor (sIL-6R), IL-12(total), IL-13, MIP-1 $\beta$ , MCP-1, and/or RANTES.

[031] "Expression" refers to transcription and translation occurring within a host cell. The level of expression of a DNA molecule in a host cell can be determined on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of DNA molecule encoded protein produced by the host cell (Sambrook *et al.*, 1989, Molecular cloning: A Laboratory Manual, 18.1-18.88).

[032] "Genetically engineered" refers to any recombinant method used to create a eukaryotic host cell that expresses a protein of interest. Methods and vectors for genetically engineering host cells are well known; for example, various techniques are illustrated in Current Protocols in Molecular Biology, Ausubel *et al.*, eds. (Wiley & Sons, New York, 1988, and quarterly updates). Genetic engineering techniques include, but are not limited to, expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Pat. No. 5,272,071 to Chappel) and transactivation by engineered transcription factors (see, for example, Segal *et al.*, 1999, Proc Natl Acad Sci USA 96(6):2758-63).

[033] As used herein, the term "high molecular weight" depends upon the particular macromolecular sample (*e.g.*, biopolymer) to be tested but generally refers to a molecular weight greater than  $1 \times 10^6$  daltons. A high molecular weight

HA viscoelastic biopolymer is generally in the range of  $1 \times 10^6$  to  $1 \times 10^7$  daltons (more particularly, in the range of  $2.5 \times 10^6$  to  $5.0 \times 10^6$  daltons).

[034] As used herein, the term "hyaluronic acid" and "sodium hyaluronate" are used interchangeably and refer to the acid and/or sodium salt of "hyaluronan." Hyaluronan is a non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is one of the chief components of the extracellular matrix, contributes significantly to cell proliferation and migration, and may also be involved in the progression of some malignant tumors. Hyaluronan is a polymer of disaccharides themselves, composed of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating  $\beta$ -1,4 and  $\beta$ -1,3 glycosidic bonds. Hyaluronan can be 25,000 disaccharide repeats in length. Polymers of hyaluronan can range in size from 5,000 to 20,000,000 Da *in vivo*. The average molecular weight in human synovial fluid is 3–4 million Da, and hyaluronan purified from human umbilical cord is approximately 3,140,000 Da.

[035] "Inactivated" refers an activity that has been rendered nonfunctional.

[036] As used herein, the term "inflammatory activity" refers to any agent that causes, or tends to cause, inflammation in an animal subject to which it is administered. Inflammation is a local response to cellular injury or noxious agent that is marked by capillary dilation, leukocytic infiltration, redness, heat, pain, and swelling.

[037] "Isolated" refers to a polynucleotide or polypeptide that has been separated from at least one contaminant (polynucleotide or polypeptide) with which it is normally associated. For example, an isolated antibody or an isolated polynucleotide is in a context or in a form that is different from that in which it is found in nature.

[038] "Polynucleotide" refers to a sequence of nucleotides. The nucleotides are either a sequence of polyribonucleotides or polydeoxyribonucleotides, or a mixture of both. Examples of polynucleotides in the context of the present invention include single and double stranded DNA, single and double stranded RNA, and hybrid molecules that have both mixtures of

single and double stranded DNA and RNA. Further, the polynucleotides of the present invention can include one or more modified nucleotide.

[039] As used herein the term "protein" and "polypeptide" are used interchangeably and is considered to be any chain of at least ten amino acids linked by peptide bonds. Purification of a protein from contaminating proteins can be accomplished through any number of known techniques, including, ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Various protein purification techniques are illustrated in Current Protocols in Molecular Biology, Ausubel *et al.*, eds. (Wiley & Sons, New York, 1988, and quarterly updates).

[040] The percent identity of one polynucleotide, polypeptide or protein in relation to another may be determined by visual inspection and mathematical calculation, or by a comparison of two sequences using various computer programs used by those of skill in the art. For example, the percent identity of two sequences can be determined using the GAP computer program, based on the algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482-489, (available from the University of Wisconsin Genetics Computer Group (UWGCG), University Research Park, Madison, Wis.). The preferred default parameters for the GAP program include: (1) a scoring matrix, *blosum62*, as described by Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89:10915 ); a gap weight of 12; (3) a gap length weight of 4; and (4) no penalty for end gaps. Other programs used by those skilled in the art of sequence comparison may also be used.

[041] "Vector," "extra-chromosomal vector", or "expression vector" refers to a first polynucleotide molecule, usually double-stranded, which can have inserted into it a second polynucleotide molecule, for example a heterologous polynucleotide. A heterologous polynucleotide may or may not be naturally found in the host cell, and can be one or more additional copy of a nucleic acid sequence naturally present in the host genome. The vector transports the foreign polynucleotide into a suitable host cell. Once in the host cell, the vector can be capable of integrating into the host cell chromosomes. The vector can also

contain the necessary elements to select cells containing the integrated polynucleotide, as well as elements to promote transcription of mRNA from the transfected polynucleotide. Examples of vectors that may be adapted for use in certain embodiments of the present invention include, but are not limited to, plasmids, bacteriophages, cosmids, retroviruses, and artificial chromosomes.

[042] Unless otherwise stated, the techniques utilized can be found in any of several well-known references, such as: *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.* (1989) *Molecular cloning: A Laboratory Manual*), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel (1991) Academic Press, San Diego, Calif.), "Guide to Protein Purification" in *Methods in Enzymology* (M. P. Deutscher, 3d., (1990) Academic Press, Inc.), *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.* (1990) Academic Press, San Diego, Calif.), *Culture of Animal Cells: A Manual of Basic Technique*, 2<sup>nd</sup> ed. (R. I. Freshney (1987) Liss, Inc., New York, N.Y.), and *Gene Transfer and Expression Protocols*, pp 109-128, ed. E. J. Murray, The Humana Press Inc., Clifton, N.J.).

#### Pro-Inflammatory Cytokines and Cell-Derived Mediators of Inflammation

[043] The presence of a pyrogen or other inflammatory activity in a test sample stimulates blood monocytes, as well as other leukocytes, to produce and release these endogenous pyrogenic mediators of the inflammatory response. The invention provides methods for detecting one or more inflammatory activities, or pyrogens, in a sample by measuring the production and release of endogenous pyrogenic mediators of the inflammatory response, including cytokines (*e.g.*, IL-6, TNF- $\alpha$  and/or IL-1 $\beta$ ).

[044] The pro-inflammatory cytokine IL-6 is particularly useful in the method of the invention, however any endogenous mediator of the inflammatory response that is detectable may be used as the basis of the pyrogen test of the present invention. Preferably, a cytokine or endothelin marker is employed because they are easy to detect (*e.g.*, by ELISA). It has been found that intraperitoneal injection of an endotoxin or non-endotoxin pyrogen produces several classes of cytokines, including, but not limited to pro-inflammatory

cytokines (IL-6, as well as TNF- $\alpha$ , IL-1, IL-6), anti-inflammatory cytokines (IL-4, IL-10, IL-13, IL-1ra, TGF), Th1 (IL-2, IFN, IL-12), Th2 (IL-4, IL-5, IL-6, IL-10, IL-13), IL-1 $\beta$ , IL-1ra, IL-8, and PGE<sub>2</sub>. Particularly useful cytokine markers for use in the invention include IL-6, TNF- $\alpha$ , IL-1 $\beta$ , IL-1ra, IL-6, IL-8 and PGE<sub>2</sub>.

[045] IL-6 is a particularly useful cytokine marker to assay in the method of the invention. IL-6 is produced in detectable amounts with a relatively short latency period. Immunoreactive IL-6, unlike immunoreactive IL-1 $\beta$  and TNF- $\alpha$ , is secreted in relatively large quantities, permitting a more complete and accurate estimation of its level in the body of the test animal, as reflective of the relative amount of inflammatory activity in the test sample. Nevertheless, TNF- $\alpha$  or IL-1 $\beta$  could also be used as a cytokine marker in the present invention. TNF- $\alpha$  is produced earlier than IL-6 in the monocyte pyrogen response. Thus, an embodiment of the invention which assays TNF- $\alpha$  would use a shorter incubation time (about 1 to 2 hours) than embodiments which assay IL-6. Different pyrogenic contaminants may elicit different cytokine responses in the cell culture. Therefore, the invention may be tailored to detect the formation of particular cytokines when contamination with a particular pyrogen which causes secretion of those cytokines is probable for a pharmaceutical product.

#### Detection Methods

[046] Once the pro-inflammatory cytokine, or other cell-derived mediator of inflammation has been determined, a specific method for detecting that cytokine is selected for use in the method of invention. Any of a number of different detection techniques may be adapted to the method of the invention.

[047] Particularly useful detection methods employ one or more antibodies specific to the analyte to be detected (e.g., the pro-inflammatory cytokine or other cell-derived mediator of inflammation of interest). Once the particular analyte is selected, an antibody to that analyte may be obtained commercially, or by routine experimentation, for use in the method of the invention. For example, polyclonal antibodies purified under stringent conditions, (e.g., as described in Example 1 of U.S. Pat. No. 6,696,261) work well in the pyrogen test. Because the animal blood from which the polyclonal antibodies are isolated is naturally pyrogen-free (if taken

from healthy animals), one must simply prevent contamination of the raw materials with pyrogens during purification to obtain a pyrogen-free product. Pyrogen-free buffers and solid phases are used in affinity chromatography columns to obtain pyrogen-free polyclonal antibodies are known in the art. Alternatively, monoclonal antibodies from hybridoma cultures can be used. However, when using monoclonal antibodies, reasonable care must be taken to isolate the antibodies free of contaminating pyrogens which may be present in the hybridoma cell culture, particularly the pyrogen to which the assay is to be directed.

[048] For use in the present invention, the antibody to the cytokine may be applied to a surface in an assay system. Methods, such as coating, for binding antibodies onto a surface in an assay system, such as a microtiter well, are well known in the biochemical arts. Many assay systems are available commercially, and the manufacturer usually provides materials and instructions for coating antibodies onto a surface of the system. Because of their ease of reading and the small sample volume required, microtiter wells in which a portion of the interior surface of the well is coated by the antibody are used in a preferred embodiment of the present invention. In order to fully exploit the advantages of the invention, it is preferred that the microtiter well be part of a microtiter plate, which is a planar array of similar wells, situated so that the array of wells may be read with automated immunoassay plate reading equipment (see, *e.g.*, U.S. Pat. No. 5,281,540, hereby incorporated by reference). Automated equipment such as ELISA plate readers (*e.g.*, the Ultramark Microplate Reader, available from Bio-Rad Laboratories, Inc.), automate the assay evaluation process and greatly decrease the per-test cost. If desired, microtiter well plates can be rendered pyrogen-free (if not already supplied as such) by extensive washing with pyrogen-free buffer. In a particularly preferred embodiment of the present invention, anti-IL-6 polyclonal antibodies are coated onto the wells of an ELISA plate. However, other immuno-diagnostic test formats (*e.g.*, in which the antibody is coated on a dipstick or bead) are acceptable for use in the present invention.

[049] In addition to the "capture" antibody, other antibodies and reagents for use in assaying the cytokine may be applied when making microtiter plate

systems for use in the present invention. For instance, after the capture antibody is applied to the microtiter plate, the remaining binding sites of the plate can be "blocked" with another protein. After blocking, a labeled detection antibody (such as a biotinylated or enzyme-labeled antibody) can be applied to the microtiter plate, along with a protective glazing compound. Thus, when a sample is incubated in the microtiter well, as described below, a released cytokine is captured by the capture antibody bound to the well and labeled by the detecting antibody simultaneously during the sample incubation period.

[050] It is important to note that when employing the methods of the invention, the level of cytokine or other cell-derived inflammatory mediator may be measured using any of a broad number of techniques available to the person of ordinary skill in the art, *e.g.*, direct physical measurements (*e.g.*, mass spectrometry) or binding assays (*e.g.*, immunoassays, agglutination assays, and immunochromatographic assays). The method may also comprise measuring a signal that results from a chemical reaction, *e.g.*, a change in optical absorbance, a change in fluorescence, the generation of chemiluminescence or electrochemiluminescence, a change in reflectivity, refractive index or light scattering, the accumulation or release of detectable labels from the surface, the oxidation or reduction or redox species, an electrical current or potential, changes in magnetic fields, *etc.* Suitable detection techniques may detect binding events by measuring the participation of labeled binding reagents through the measurement of the labels via their photoluminescence (*e.g.*, via measurement of fluorescence, time-resolved fluorescence, evanescent wave fluorescence, up-converting phosphors, multi-photon fluorescence, *etc.*), chemiluminescence, electrochemiluminescence, light scattering, optical absorbance, radioactivity, magnetic fields, enzymatic activity (*e.g.*, by measuring enzyme activity through enzymatic reactions that cause changes in optical absorbance or fluorescence or cause the emission of chemiluminescence). Alternatively, detection techniques may be used that do not require the use of labels, *e.g.*, techniques based on measuring mass (*e.g.*, surface acoustic wave measurements), refractive index (*e.g.*, surface plasmon resonance measurements), or the inherent luminescence of an analyte.

[051] Binding assays for measuring cytokine levels may use solid phase or homogenous formats. Suitable assay methods include sandwich or competitive binding assays. Examples of sandwich immunoassays are described in U.S. Pat. No. 4,168,146 and U.S. Pat. No. 4,366,241. Examples of competitive immunoassays include those disclosed in U.S. Pat. No. 4,235,601, U.S. Pat. No. 4,442,204, and U.S. Pat. No. 5,208,535.

[052] Multiple cytokines may be measured using a multiplexed assay format, *e.g.*, multiplexing through the use of binding reagent arrays, multiplexing using spectral discrimination of labels, multiplexing by flow cytometric analysis of binding assays carried out on particles (*e.g.*, using the Luminex system). Suitable multiplexing methods include array based binding assays using patterned arrays of immobilized antibodies directed against the cytokines of interest. Various approaches for conducting multiplexed assays have been described. For example, multiplexed testing is described in U.S. Pat. Publ. Nos. 20040022677 and US20050052646, as well as, U.S. Pat. Publ. No. 20030207290, U.S. Pat. Publ. No. 20030113713, U.S. Pat. Publ. No. 20050142033; and U.S. Pat. Publ. No. 20040189311. One approach to multiplexing binding assays involves the use of patterned arrays of binding reagents (see, *e.g.*, U.S. Pat. Nos. 5,807,522 and 6,110,426, Delehanty (2004) Methods Mol. Biol. 278:135-44; Lue *et al.* (2004) Methods Mol. Biol. 278: 85-100; Lovett (2000) Science 289: 536-537; Berns (2000) Nature 403: 491-492; Walt (2000) Science 287: 451-452 for more details). Another approach involves the use of binding reagents coated on beads that can be individually identified and interrogated. International Patent publication WO9926067A1 describes the use of magnetic particles that vary in size to assay multiple analytes; particles belonging to different distinct size ranges are used to assay different analytes. The particles are designed to be distinguished and individually interrogated by flow cytometry. Also available for use in the methods of the invention is a multiplex binding assay in which 64 different bead sets of microparticles are employed, each having a uniform and distinct proportion of two dyes (see Vignali, (2000) J. Immunol. Meth. 243: 243-255). Similar approaches include a method involving a set of 15 different beads of differing size and fluorescence have been disclosed as useful for simultaneous typing of multiple pneumococcal serotypes (see Park, *et al.* (2000) Clin Diagn Lab Immunol. 7: 486-

9); and a multiplex sandwich assay for simultaneous quantification of six human cytokines (see Bishop, *et al.* (1999) Clin Chem. 45:1693-1694).

[053] In general, the test method involves testing one or more samples, e.g., body fluid samples, particularly an intraperitoneal body fluid sample, from the test animal. In certain embodiments, the test methods of the invention may be conducted on a single sample from the test animal including, but not limited to, peritoneal fluid, blood, serum, plasma, hair, sweat, urine, feces, tissue, biopsies, saliva, skin, mucosa, CNS fluid, bone marrow, tissue extracts, cells, cell extracts, cell culture supernatants, and lymphatic fluids. Particularly advantageous are peritoneal fluid, blood, blood serum, blood plasma, or biopsy tissue. In addition, multiple samples from the test animal may be tested for one or more inflammatory cytokines and/or other cell-derived mediators of inflammation using any of the preceding detection methods.

#### Test Sample Preparations

[054] The invention provides methods of detecting an inflammatory activity in a test sample, particularly a test sample primarily composed of a nonimmunogenic, noninflammatory agent such as hyaluronan or other biopolymer. Suitable test samples including preparations of polysaccharides, including homopolysaccharides and heteropolysaccharide for use in biological applications such as in medical implants or as biologic agents. An exemplary heteropolysaccharide containing test sample for use in the method of the invention is a glycosaminoglycan (GAG) test sample. Glycosaminoglycans especially suitable for use in the present invention are hyaluronic acid (HA), chondroitin sulfate, dermatan sulfate, keratan sulfates, heparin and heparan sulfate. The biopolymer may be obtained from a biological source, or it may be a product of *in vitro* enzymatic or chemical synthesis, or combinations thereof. A biological source may be a bacterial, yeast, plant, amphibian, avian or mammalian organism. For example, HA may be isolated from any of fermented *Streptococcal* cultures, human umbilical cords, bovine cartilage or rooster combs. Enzymatic *in vitro* enzymatic synthesis of polysaccharides including HA, cellulose, polymannuronic acid and chitin is described in WO 95/24497. The use of a recombinantly produced hyaluronan synthase enzyme for *in vitro* synthesis of HA

is described in U.S. Pat. No. 6,602,693. HA obtained from any of the aforementioned sources may be subsequently chemically modified or derivatized as described for example in U.S. Pat. No. 4,851,521, U.S. Pat. No. 4,713,448, U.S. Pat. No. 5,336,767 and U.S. Pat. No. 5,099,013.

[055] HA is a naturally occurring biopolymer consisting of repeating disaccharide units of D-glucuronic acid in  $\beta$ -(1-3) linkage with N-acetyl-D-glucosamine, wherein each disaccharide unit is connected to its adjoining neighbors by  $\beta$ -(1-4) linkages. The salt sodium hyaluronate (NaHA) is found at physiological pH in human and vertebrate joint synovial fluid, connective tissue, vitreous humor of the eye and healthy skin tissue, and is an extracellular secretion product of several bacterial species, particularly of the genus *Streptococcus*. The method of the invention is particularly suitable for HA. NaHA has been isolated and purified from microorganisms and vertebrates. Bacterially-derived NaHA may be derived from fermentation of *Streptococcus zooepidemicus*, e.g. particularly a hemolysin negative mutant such as HA-116 (ATCC 239920) as described in further detail in U.S. Patent Nos. 4,780,414 and 4,784,990. The term hyaluronic acid (HA) means hyaluronic acid, salts thereof, such as sodium, potassium, magnesium, calcium, lysine, ammonium, triethanolamine and propanolamine hyaluronates, metal salts thereof, such as cobalt, zinc, copper, iron, manganese and lithium hyaluronate, and chemically modified and derivatized forms thereof, as disclosed for example in U.S. Pat. No. 4,851,521, U.S. Pat. No. 5,099,013, U.S. Pat. No. 5,336,767 and U.S. Pat. No. 6,017,901.

[056] Major medical applications of manufactured NaHA products include ophthalmic surgery for cataracts and intraocular lens implantation, and in viscoelastic supplementation for the treatment of osteoarthritis in humans and large mammals. Viscoelastic supplementation, particularly in the knee, is aimed at restoring the normal rheological homeostasis of the joint network and for providing immediate protection, lubrication, shock absorption, hydrodynamic resistance and a mechanochemical barrier against stress. Intra-articular injections of NaHA have been shown to improve function and mobility and decrease pain. More recent medical applications of manufactured NaHA include medical device coatings, surgical adhesion prevention products, drug delivery

vehicles, bone replacement materials and wound healing materials. Marketed NaHA products include BioLon™, Biolon™ Prime, BioHy™ (all from BTG), Hyalart™ (Fidia), Synvisc™ Hyalan G-F 20 (Biomatrix), Healon™ (Pharmacia) BD Visc™ (Becton Dickinson) and Orthovisc™ (Anika Therapeutics).

[057] Additional viscoelastic biopolymers which are the products of bacterial fermentation include for example curdlan gum ( $\beta$ -1-3-D-glucan), produced by *Alcaligenes faecalis*, gellan gum (tetrasaccharide backbone of L-rhamnose and D-glucose with glyceryl and acetyl substituents) produced by *Sphingomonas elodea* and xanthum gum ( $\beta$ -1-4-D-glucan with mannose and glucuronic acid side chains), produced by *Xanthomonas campestris*.

[058] The viscoelastic biopolymer suitable for the method of the invention may be a homopolysaccharide *i.e.* assembled from a single type of monosaccharide, or a heteropolysaccharide *i.e.* assembled from two or more different types of monosaccharides. Examples of homopolysaccharides include carboxymethylcellulose, chitin, polymannuronic acid, curdlan gum, scleroglucan and dextran. Examples of heteropolysaccharides include glycosaminoglycans, alginates, carageenans, guar gum, pectins, locust bean gum and xanthum gum. The method of the invention is particularly suitable for glycosaminoglycans, also known as acid mucopolysaccharides, which are composed of repeating disaccharide units in which one of the two monosaccharides is always either N-acetylglucosamine or N-acetylgalactosamine. Examples of glycosaminoglycans include HA, chondroitin, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, dermatan sulfate, keratan sulfate, heparin and heparan sulfate. Both homo- and heteropolysaccharides may be linear or branched structures. The component monosaccharides of a biopolymer may be released by acid hydrolysis and detected by analytical techniques such as thin layer chromatography and/ or high pressure liquid chromatography. Suitable viscoelastic biopolymers may be within a wide range of molecular weight (*e.g.*,  $1 \times 10^4$  to  $1 \times 10^7$  daltons). A bulk manufactured and purified viscoelastic biopolymer is generally sterilized, *e.g.* by filter sterilization, before it is tested for inflammatory activity by the method of the invention.

[059] The foregoing detailed description includes many specific details. The inclusion of such detail is for the purpose of illustration only and should be understood not to limit the invention. In addition, features in one embodiment may be combined with features in other embodiments of the invention. The patent and scientific literature referred to in this description establishes knowledge that is available to those of skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the invention contained in the specification, the specification will supersede any contradictory material.

### **Examples**

[060] This invention is further illustrated by the following examples, which should not be construed as limiting.

#### **Example 1: Cell-Dependent Oxidative Burst Inflammatory Test**

[061] A mouse-based cell-dependent oxidative burst test has been used successfully to assay for biologically active inflammatory impurities in hyaluronic acid and other heteropolysaccharides and implanted materials. In this assay, an acute inflammatory response is initiated by intraperitoneal (ip) injection of unpurified HA into the mouse, but is not initiated by injection of highly purified HA.

[062] Two parameters indicate that an inflammatory process has occurred in the injected mouse: elevation of the total number of peritoneal exudate cells (PEC); and high oxidative burst (OB) activity of the isolated PEC *in vitro*. Cell infiltration and accumulation at the injected site follows a specific time course, and is the initial step of the local inflammatory response. The increase in oxygen uptake and the consequent release of oxygen metabolites, primarily superoxide radical ( $O_2^-$ ), is particularly high in polymorphonuclear leukocytes (PMNs), the primary responders to inflammatory material. In PMN's, the oxidative burst is amplified by addition of phorbol myristate acetate (PMA) to PEC in a typical dose-dependent manner.

[063] In order to determine if new NaHA fermentation batches contain inflammatory impurities, three experimental groups are used: a positive control group and a negative control group, and a test sample group. Since the local inflammatory response is complex and varies as a function of different stimulants, partially purified hyaluronate was used as the inflammatory reference material (positive control) in the assay. The negative control (NaHA reference standard dissolved in saline) served to confirm proper execution of the test procedure and the quality of the test animals. The test NaHA sample was prepared as a 1% solution in saline. The NaHA reference standard was used as the negative control. Mice of the (BALB/c x C57B1) F<sub>1</sub> inbred strain were obtained from Jackson Laboratories (Bar Harbor, Maine). Male mice 1-2 months of age (20-22g) were used for the test and were kept for 5 days prior to being used in the assay to insure adequate adjustment to their new environment.

[064] The following assay reagents were prepared in advance: Ferricytochrome c type III (Sigma, cat. no. C-2506); tissue culture grade dimethylsulfoxide (DMSO) (Sigma, Cat. No. D2650); phorbol myristate acetate (PMA, or TPA, Sigma, cat. no. P-8139) dissolved to a concentration of 2 mM and serially diluted to 0.2 mM, 200 nM, and 2 nM stocks.

[065] The test consists of three groups: group I, the negative control, consisting of 10 mice injected with NaHA reference standard dissolved in sterile saline; group II, the positive control, consisting of 3 mice injected with inflammatory (unrefined) NaHA; and group III, the experimental group, consisting of 15 mice injected with test samples.

[066] A 1% NaHA solution of the test samples, negative control sample and positive control (inflammatory reference) sample, were prepared by dissolving bulk NaHA at a concentration of 11.5 mg/ml saline (to compensate for water and salt in the sample). The same saline solution should be used for dissolution of the negative control and the test sample.

[067] The complete dissolution of the NaHA was checked by visual inspection. When dissolution was complete, the control and test samples were diluted 1:10 and agitated on a shaker for one hour. The NaHA concentration was

measured polarimetrically using NaHA specific rotation polarimetry measurements. The concentration was confirmed to be  $10 \pm 1$  mg/ml NaHA.

[068] The test samples, negative control and positive control were loaded into 5 ml syringes and sterile 23G needles were attached to the syringes. Mice were injected at about the center of the lower left quadrant of the abdomen by holding the syringe at an ~20 degree angle to the skin, towards the chest. Each mouse was injected with 1.0 ml of sample while being careful not to damage internal organs.

[069] At 16-18 hours after injection, the mice were sacrificed by cervical dislocation, and the peritoneal exudate cells (PEC) by rinsing the peritoneal cavity with 15 ml of ice-cold Hank's balanced salt solution, and collecting the wash in 50 ml centrifuge tubes. The peritoneal cavity was then washed with an additional 15 ml of Hank's solution, which was added to the first wash. The tubes were numbered in the order that the cells were harvested in, and kept on ice at all times.

[070] The exudate was centrifuged at 10°C at 1400 rpm for 10 minutes and the supernatant was discarded. The cell pellets were visually inspected and hemolytic (bloody pellets) were discarded and replaced with PEC from one or more of the reserved mice. The cell pellets were divided from each test group according to size (*i.e.*, tubes containing large cell pellets and small cell pellets). The tubes were further divided into four subgroups (4 mice each), two subgroups of four mice each for the negative control and three subgroups of four mice each for the test samples.

[071] The cell pellets were resuspended in 1 ml Hank's salt solution and the cells of each subgroup were pooled in one test tube. The cell pellets of the positive control groups can be pooled together without subdividing. The samples were centrifuged and gently resuspended in 1 ml of Hank's solution.

[072] The resuspended cell pellets were diluted in duplicate to yield samples between  $10^4$  and  $10^5$  cells/ml (this required diluting the PEC of the negative control and the test sample between 1:3 to 1:10 and those of the positive control between 1:50 to 1:200). Each dilution was counted in duplicate with a

hemocytometer by counting at least two different fields, with each field containing 16 subdivisions, of the hemocytometer and calculating the cell number per ml by multiplying the mean result by the dilution factor and by  $10^4$ .

[073] To measure the oxidative burst activity of the PEC cells, ferricytochrome C solution was dissolved to a final concentration of  $160 \mu\text{M}$  ( $2 \text{ mg/ml}$ ) in Hank's buffer. The cells were suspended in Hank's containing  $80 \mu\text{M}$  freshly dissolved ferricytochrome C at a concentration of  $1 \times 10^5$  cells/ml, and 1 ml aliquots were dispensed, 6 wells per group, into 24-well microtiter plates. PMA was added at concentrations of 0 nM, 2 nM and 200 nM, in a volume of 10 ml per well, using duplicate wells for each concentration. The stock solution of 2 mM PMA in DMSO serves for the serial dilutions and DMSO only is also applied to the wells as a control. The 24-well plates were then placed at  $37^\circ\text{C}$  (5%  $\text{CO}_2/\text{air}$ ) incubator for 60 minutes.

[074] The reduction of cytochrome C was quantified by measuring the absorbance of each well solution at 550 nm against a blank of Hank's solution containing  $80 \mu\text{M}$  ferricytochrome C, but without PEC. The results were graphed by plotting the oxidative burst ( $A_{550 \text{ nm}}$ ) as a function of PMA concentration in each culture. The weighted average oxidative burst for each subgroup was calculated by multiplying the oxidative burst at 200 nM PMA measured for each individual test subgroup by the number of mice in that subgroup; the sum of the values was calculated and divided by the total number of mice for all of the subgroups in the group.

[075] An acceptable test sample (*i.e.*, one without significant inflammatory activity) met the following requirements: the plots obtained showed the typical shape, *i.e.*, an increase in the  $A_{550}$  in samples containing PMA; the negative control group had an oxidative burst of  $\leq 0.10$  ( $A_{550}$ ), and the positive control had an oxidative burst of 0.15-0.40; while the oxidative burst of the test sample was  $\leq 0.10$  ( $A_{550}$ ).

#### Example 2: IL-6 Inflammatory Test of Sodium Hyaluronate (NaHA) Bacterial Fermentation Product

[076] This example summarizes the validation of an ELISA method to determine the absence of inflammation triggering response in NaHA by quantitating interleukin-6 (IL-6) concentrations in cell-free peritoneal fluid supernatant. High molecular weight NaHA is reported to be a non-pyrogenic non-inflammatory material. Nonetheless, international safety standards for use of NaHA as a medical device or biologic requires demonstration that the NaHA produced is free of inflammatory triggering components. The example demonstrates successful inflammatory activity testing of NaHA prepared by extensive chemical purification of a batch fermentation product of *Streptococcus zooepidemicus* (see U.S. Pat. Nos. 4,780,414 and 4,784,990).

[077] Male Balb/c mice, 34-36 days old weighing 20-22 Grams were obtained from Jackson Laboratories (Bar Harbor, Maine). Following receipt, the animals were kept for five days to ensure adequate adjustment to their new environment prior to being used in an inflammatory assay. Group I, the negative control, consisted of 10 mice injected with NaHA reference standard dissolved in sterile saline. Group II, the positive control consisted of 3 mice injected with a previously characterized inflammatory NaHA preparation. Group III, the experimental group, consisted of 15 mice injected with a test lot of purified NaHA product.

[078] A 1% solution of NaHA solution of the test sample, negative control sample and positive control (inflammatory reference) sample was prepared by dissolving bulk NaHA at a concentration of 11.5 mg/ml (w/w) in saline by agitating on a shaker for 2-3 days at 4°C. The same saline solution was used for dissolution of the negative control, the positive control and the test samples. The final concentration, 10 mg/ml, was confirmed by measuring a 1:10 dilution of the final NaHA preparation polarimetrically

[079] The positive control, an inflammatory NaHA preparation, was prepared from an unrefined preparation of *Streptococcus zooepidemicus* fermentation product. The negative control, non-inflammatory NaHA preparation was a preparation that had been previously tested and confirmed free of inflammatory activity by both the oxidative burst (OB) and IL-6 induction assays.

All samples were loaded into 5 ml syringes and sterile 23G needles were attached.

[080] Mice were injected with 1.0 ml of the test sample, positive control or negative control directly into the abdominal cavity, while being careful not to damage internal organs. At six hours post-injection the mice were sacrificed by cervical dislocation or exposure to CO<sub>2</sub>. A lavage from the peritoneal cavity was prepared by flushing with 3 ml of PBS, and the resulting exudate was centrifuged at 1400 rpm for 10 minutes at 10°C to remove cells and debris. The supernatants were transferred to new tubes and kept overnight at 2-8°C before measuring IL-6 levels by ELISA. The negative control and test sample supernatants were diluted 10:1 in PBS x10 - BSA 10% (9 parts supernatant with 1 part of PBS x10 - BSA 10%). The positive control supernatant was diluted 1:2 in PBS followed by dilution 10:1 in PBS x10 - BSA 10% (9 parts supernatant with 1 part of PBS x10 - BSA 10%).

[081] IL-6 ELISA's were performed using a commercial kit under conditions prescribed by the manufacturer (BD OptEIA mouse IL-6 ELISA kit, Becton Dickinson Biosciences, San Jose, CA). The wells of a 96-well microtiter plate coated with an IL-6 monoclonal capture antibody were washed with 1x PBS + 0.05% Tween-20 (PBST) and 200 µl of the test, negative or positive control sample was added to each well. In addition a standard curve was established using 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/ml dilutions of IL-6. The microtiter plate was sealed and incubated for 2 hours at room temperature. The samples were removed by aspiration, the wells were washed and the amount of IL-6 captured on the surface of the plate was quantitated by adding a streptavidin-horseradish peroxidase (Avidin HRP) conjugate mixed with biotinylated anti-mouse IL-6 antibody to produce antibody-(IL-6 antigen)-antibody "sandwich". The microtiter wells were washed 7 times before adding 3, 3', 5, 5'-tetramethylbenzidine (TMB) colorimetric indicator in buffered saline to each well. The plate was incubated at room temperature and the optical density at 405 nm using the ELISA plate reader. When the OD of the highest IL-6 concentration of the standard curve was between 1.0-1.2, the reaction was stopped by adding 100

ml of HRP stop solution per well. The absorbance of the wells was measured using the Multicalc protocol for the detection of IL-6 using BD's kit.

[082] A nonparametric Mann-Whitney test was used to statistically analyze the results. Prior to statistical analysis, the mean of IL-6 concentration values was determined and all IL-6 concentration values derived from the ELISA were transformed to ln (natural log). The negative control and positive control groups were compared by the Mann-Whitney non-parametric test. A  $p < 0.05$  value indicates a significant difference between the positive control and the negative control. Next, the positive control and the test sample were compared using the Mann-Whitney nonparametric test. A  $p < 0.05$  value indicates a significant difference between the positive control and the test sample. Finally, the negative control and the test sample groups were compared by the Mann-Whitney non-parametric test. A  $p > 0.05$  indicates no significant difference between the negative control and the test sample. The test samples were determined to be acceptable based upon the following criteria: the positive control versus the test sample gave a  $p$  value  $< 0.05$ , indicating a significant difference between the positive control and the test samples; and both the negative control and the test sample groups are uniformly homogenous and the same ELISA result is obtained, or the difference between the negative control and the test sample provided a  $p$  value of  $> 0.05$ , which indicates that the difference is not statistically significant.

### Example 3: Comparison of IL-6 and Cell-Dependent Oxidative Burst Inflammatory Tests

[083] The cell-dependent oxidative burst (OB) test of cells harvested from the peritoneum of NaHA-injected mice was validated against the owl-monkey inflammatory assay. A review of historical test results shows that the OB test method has a narrow signal-to-noise ratio, a tendency to yield hemolytic samples (obtained frequently upon animal handling and which have to be excluded from the test) and is limited to the number of cells withdrawn from individual mouse peritoneum available for OB testing. As described above, a modified test method which measures interleukin-6 (IL-6) concentrations by ELISA in a cell-free peritoneal fluid supernatant was validated. Test results were subjected to

statistical analysis by nonparametric Mann-Whitney test; results were tested for statistically significant differences to the positive controls. Animal handling, NaHA administration, and peritoneal fluid withdrawal remained unchanged in the modified test procedure. This example describes how comparability between the two test methods was determined in order to support the replacement of the OB method by the IL-6 ELISA method for determination of the inflammation response in NaHA batches.

[084] The OB and IL-6/ELISA test methods were performed essentially as described in examples 1 and 2, respectively, above. Test results after transformation to the natural logarithm (ln) were subjected to statistical analysis by the nonparametric Mann-Whitney test; results were tested for statistically significant differences to the positive controls and for statistically non-significant differences to the negative controls.

[085] Tables 1 and 2 summarize the modified inflammation assay (IL-6 ELISA method) performance and results.

[086] Table 1 shows the values of IL-6 concentrations (transformed to the natural logarithm (ln)) in peritoneal fluid of each mouse. Most of the negative control and test sample values were below the minimal detection concentration (MDC) and therefore are noted as the MDC value.

**Table 1**

	IL-6 Concentration (pg/ml) in Peritoneal Fluid								MDC
PC	8.133	8.294	8.294	8.256	8.264	--	--	--	4.100
NC	4.100	4.100	4.100	4.100	4.100	4.100	4.100	4.100	
TS #021	4.100	4.537	4.100	4.100	4.100	4.100	4.100	4.100	
TS #022	4.100	4.100	4.100	4.100	4.100	4.100	4.100	4.100	
TS	4.909	4.100	4.100	4.100	4.100	4.100	4.100	4.100	

#023									
PC	8.294	8.294	8.294	8.280	8.294	--	--	--	2.774
NC	2.774	2.774	2.774	2.774	2.774	2.774	2.774	2.774	
TS #24	2.774	2.774	2.774	2.774	2.774	2.774	2.774	2.774	
TS #25	2.774	2.774	2.774	2.774	2.774	2.774	2.774	2.774	
PC	7.040	6.644	7.031	6.442	6.797	--	--	--	3.371
NC	3.371	3.371	3.371	3.371	3.371	3.371	3.371	3.371	
TS #14	3.371	3.371	3.371	3.371	3.371	3.371	3.371	3.371	
TS #1	4.089	4.965	3.371	4.098	3.371	4.134	3.371	4.567	
TS #2	3.371	3.371	3.371	3.371	3.371	3.371	3.371	3.371	
PC	6.789	6.349	7.062	6.700	7.071	--	--	--	2.778
NC	2.778	2.778	2.778	2.778	2.778	2.778	2.778	2.778	
TS #4	2.778	3.673	3.466	3.575	2.778	4.118	2.778	2.778	
TS #5	2.778	2.778	4.283	4.019	4.560	2.778	4.816	2.778	

[087] Table 2 shows the statistical analysis of the comparison between the positive control (PC) and negative control (NC) and test samples (TS), and NC vs. TS. Significant differences were obtained between the PC and NC and between the PC and TS, while no statistically significant differences were obtained between the PC and NC and between the PC and TS. No statistically significant differences were observed between the NC vs. TS samples. All test batches met the IL-6 ELISA method requirements.

**Table 2 (Statistical Analysis of Experiments)**

	PC vs. NC	PC vs. TS	NC vs. TS	Statistical Significance Criteria Met
P value criteria	< 0.05	< 0.05	> 0.05	n.a.
TS #21		0.001	1.000	Yes

TS #22	0.000	0.000	1.000	Yes
TS #23		0.001	1.000	Yes
TS #24		0.000	1.000	Yes
TS #25	0.000	0.000	1.000	Yes
TS #13		0.002	0.727	Yes
TS #1	0.000	0.000	1.000	Yes
TS #2		0.000	1.000	Yes
TS #4		0.002	0.125	Yes
TS #5	0.000	0.002	0.125	Yes

[088] Inflammation testing according to the OB method was carried out as part of the testing of the ten bulk NaHA batches. These results are summarized in Table 3. All of these test batches met the specification for the OB test inflammation assay.

**Table 3 (Oxidative Burst Results)**

	<b>A<sub>550</sub></b>	<b>Statistical Significance Criteria Met</b>
Sample Limit	$\leq 0.1$	n.a.
TS #21	0.05	Yes
TS #22	0.07	Yes
TS #23	0.06	Yes
TS #24	0.03	Yes
TS #25	0.04	Yes
TS #13	0.06	Yes
TS #1	0.08	Yes
TS #2	0.07	Yes
TS.#4	0.04	Yes
TS #5	0.05	Yes

[089] A standard operating procedure for testing bacterially fermented batches of purified HA for use in synovial fluid supplementation utilizes 8 animals for the negative control, 8 animals for the test sample and 5 animals for the positive control.

[090] The patent and scientific literature referred to in this description establishes knowledge that is available to those of skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the invention contained in the specification, the specification will supersede any contradictory material.

[091] The foregoing detailed description includes many specific details. The inclusion of such detail is for the purpose of illustration only and should be understood not to limit the invention. The specific embodiments described herein are offered by way of example only and are not meant to be limiting in any way. In addition, features in one embodiment may be combined with features in other embodiments of the invention. Indeed, many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art.

[092] It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the claims, which follow.

## Claims

1. A method of detecting inflammatory activity in a sample comprising:  
administering a test sample to an animal; and  
detecting a level of interleukin-6 (IL-6) in the animal,  
wherein an elevated level of IL-6 in the test animal, as compared to a control animal injected with a non-inflammatory control sample, indicates that the test sample has inflammatory activity.
2. The method of claim 1, wherein the test sample is a biological sample.
3. The method of claim 1, wherein the test sample is a medical device.
4. The method of claim 1, wherein the test sample is a biopolymer.
5. The method of claim 1, wherein the test sample is a heteropolysaccharide selected from the group consisting of hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin sulfate, heparin, heparan sulfate, agar, alginate, carrageenan, gellan, guar gum, locust bean gum and xanthan gum.
6. The method of claim 5, wherein the heteropolysaccharide is produced by bacterial fermentation.
7. The method of claim 6, wherein the heteropolysaccharide is produced by bacterium of the genus *Streptococcus*.
8. The method of claim 1, wherein the inflammatory activity detected is a *Streptococcal* mucopeptide.
9. The method of claim 1, wherein the inflammatory activity detected is a bacterial endotoxin.
10. The method of claim 1, wherein the inflammatory activity detected is a lipopolysaccharide.

11. The method of claim 1, wherein the inflammatory activity detected is from a Gram-positive bacterium.
12. The method of claim 11, wherein the inflammatory activity detected is lipoteichoic acid (LTA).
13. The method of claim 1, wherein the test animal is a mammal.
14. The method of claim 13, wherein the mammal is selected from the group consisting of a mouse, a guinea pig, and a rat.
15. The method of claim 13, wherein the test sample is administered by injecting it into the abdomen of the mammal.
16. The method of claim 1, wherein the level of IL-6 is detected in a peritoneal body fluid.
17. The method of claim 1, wherein the level of IL-6 is detected with an IL-6 antibody.
18. The method of claim 1, wherein the level of IL-6 is detected with a bioassay that is sensitive to IL-6.
19. The method of claim 17, wherein the level of IL-6 is detected using an antibody-antigen-antibody sandwich enzyme-linked immunosorbent assay (ELISA).
20. The method of claim 1, wherein the level of IL-6 in the animal is determined about 4-16 hours after administering the test sample.
21. A method of detecting an exogenous non-endotoxin pyrogen in a Gram-positive bacterial fermentation product comprising:
  - injecting a mouse with the Gram-positive bacterial fermentation product intra-peritoneally;
  - collecting a peritoneal fluid from the injected mouse at least about 6 hours after injecting the Gram-positive bacterial fermentation product;
  - and

measuring the IL-6 concentration in a cell free supernatant of the peritoneal fluid, wherein an exogenous non-endotoxin pyrogen is present in the Gram-positive bacterial fermentation product if the IL-6 concentration is higher than the concentration of IL-6 in a control mouse injected with a non-inflammatory negative control.

22. The method of claim 21, wherein the exogenous non-endotoxin pyrogen is a *Streptococcal* mucopeptide.

23. The method of claim 21, wherein the Gram-positive bacterial fermentation product is hyaluronic acid (HA).

24. The method of claim 23, wherein the Gram-positive bacterium is a *Streptococcus*.

25. The method of claim 24, wherein the *Streptococcus* is *Streptococcus zooepidemicus* HA-116 (ATCC 39920).

26. The method of claim 21, wherein the mouse is selected from the group consisting of a Balb/c mouse, a CB6F1 mouse, and the progeny or descendent of a cross between a Balb/c and a CB6F1 mouse.

27. The method of claim 21, wherein the peritoneal fluid is collected about 4-16 hours after injecting the Gram-positive bacterial fermentation product.

28. The method of claim 21, wherein the concentration of IL-6 is measured using an antibody-antigen-antibody sandwich enzyme-linked immunosorbent assay (ELISA).

29. The method of claim 21, wherein the concentration of IL-6 is detected with a bioassay that is sensitive to IL-6.

30. The method of claim 21, wherein there is a statistically significant higher concentration of IL-6 concentration in the mouse injected with the Gram-positive bacterial fermentation product than in the control mouse injected with the non-inflammatory negative control.

31. A method of detecting inflammatory activity in a sample comprising:

administering a test sample to an animal; and

detecting a level of a pro-inflammatory cytokine or other cell-derived mediator of inflammation in the animal,

wherein an elevated level of the pro-inflammatory cytokine or other cell-derived mediator of inflammation in the test animal as compared to a control animal injected with the non-inflammatory control sample indicates that the test sample has inflammatory activity.

32. The method of claim 31, comprising detecting the level of a pro-inflammatory cytokine selected from the group consisting of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ .

33. The method of claim 31, comprising detecting the level of a cell-derived mediator of inflammation selected from the group consisting of lysosome granules, histamine, IL-8, leukotriene B<sub>4</sub>, nitric oxide, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

34. The method of claim 1 or 31, wherein the test sample comprises a non-immunogenic heteropolysaccharide.

35. The method of claim 34, wherein the non-immunogenic heteropolysaccharide is hyaluronan.

36. The method of claim 21, wherein the bacterial fermentation product comprises a non-immunogenic heteropolysaccharide.

37. The method of claim 36, wherein the non-immunogenic heteropolysaccharide is hyaluronan.

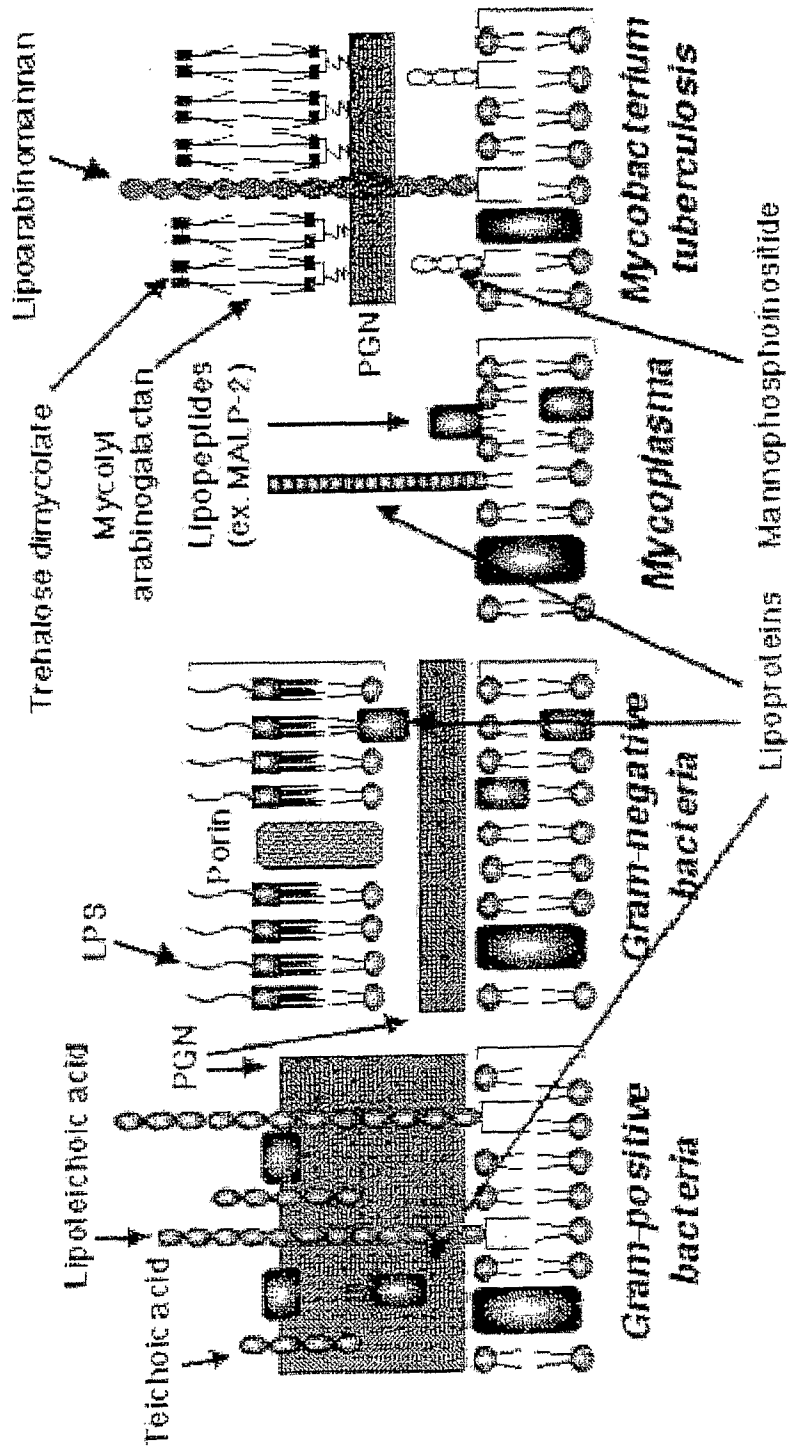
38. The method of claim 34, wherein the hyaluronan is a preparation for use in synovial fluid supplementation.

39. The method of claim 34, wherein the hyaluronan is a preparation for use as a dermatological filler.

40. The method of claim 37, wherein the hyaluronan is preparation for use in an application selected from the group consisting of synovial fluid supplementation and dermatological filling of wrinkles.

Figure 1

# Bacterial Membrane and Cell Wall Components



# Cytochrome C Test

Figure 2

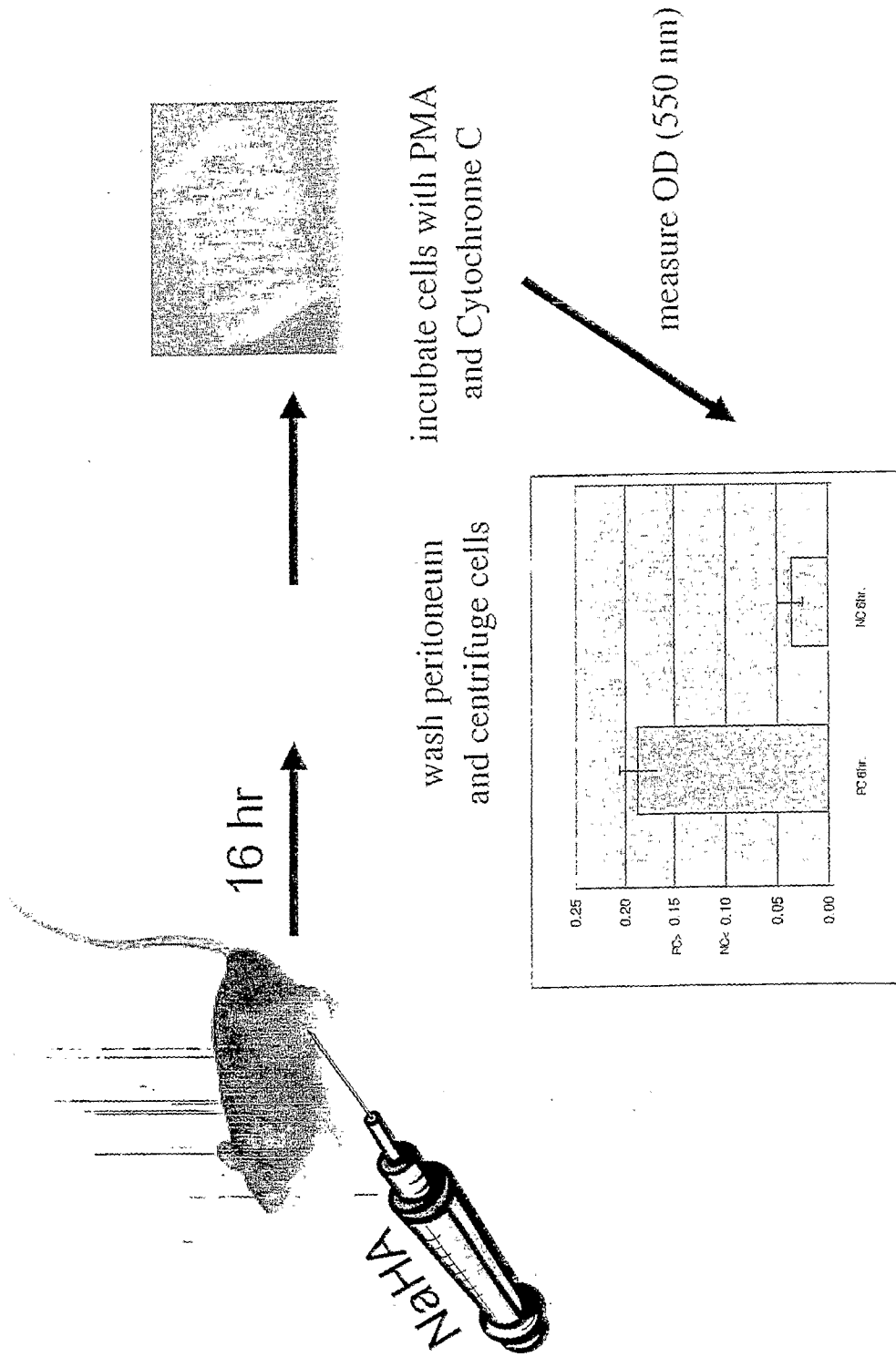


Figure 3

# IL-6 ELISA TEST

