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54	TITLE OF INVENTION

Inflammatory mediator antagonists

57	ABSTRACT (NOT MORE THAN 150 WORDS)

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The sheet(s) containing the abstract is/are attached.

If no classification is furnished, Form P.9 should accompany this form.
~~The figure of the drawing to which the abstract refers is attached.~~

A&A P208

(57) Abstract

The present invention relates to the use of an antagonist of OSM such as an antibody or small molecule in the manufacture of a medicament for the treatment or prophylaxis of an inflammatory arthropathy or inflammatory disorder, and the use of OSM in screening for such antagonists.

Inflammatory Mediator Antagonists

The present invention relates to the use of an antagonist of OSM in the manufacture of a medicament for the treatment or prophylaxis of an inflammatory arthropathy or inflammatory disorder and methods of screening for such antagonists.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects articular joints, characterised by synovial hyperplasia and extensive cellular infiltration by mononuclear cells and polymorphonuclear leukocytes(PMN). A complex, and poorly understood interplay between resident and infiltrating cell types leads to the chronic secretion of metalloproteinases(MMPs), resulting in destruction of articular cartilage, ligaments and subchondral bone (Firestein GS Current Opinion in Rheumatology. 4:348-54, 1992). Among the numerous pro-inflammatory cytokines implicated in driving RA joint pathology, TNFα has been shown to play a pivotal role, with anti-TNFα therapies showing clear benefit (Elliott MJ. et al. Lancet. 344(8930):1105-10, 1994). TNFα mediates several pathologic effects including induction of MMPs (Dayer JM. et al Journal of Experimental Medicine. 162(6):2163-8, 1985), upregulation of other pro-inflammatory cytokines (Haworth C. et al. European Journal of Immunology. 21(10):2575-9, 1991 and Dinarello CA. et al. Journal of Experimental Medicine.163(6):1433-50, 1986) and increased PMN adhesion and transendothelial cell migration(Smart SJ. Casale TB American Journal of Physiology. 266:L238-45, 1994). Though TNFα is viewed currently as the initiator of the pro-inflammatory cytokine cascade, relatively little is known of its positive regulation (Feldmann M. et al. Annual Review of Immunology. 14:397-440, 1996).

Oncostatin M (OSM) (Rose TM. Bruce AG. PNAS USA 88(19):8641-5, 1991) is a 28 kDa glycoprotein which belongs to a family of cytokines comprising IL-6, IL-11, leukaemia inhibitory factor (LIF), ciliiary neurotrophic factor (CNTF) and cardiotrophin 1 (CT-1)(Taga T. Kishimoto T. Annual Review of Immunology. 15:797-819, 1997). All members share a common signalling chain, gp130, as part of a complex family of hetero- and homodimeric receptors (Grotzinger J. et al. [Article] Proteins. 27(1):96-109, 1997). OSM shares a common heterodimeric receptor with LIF, (LIFr : gp130, type I) and also has its own unique receptor comprising OSMrβ chain and gp130 (type II) (Mosley B. et al. [Article] Journal Of Biological Chemistry. 271(51):32635-32643, 1996). OSM has long been known for effects on cell growth and differentiation (Horn D. et al [Journal Article]Growth Factors. 2(2-3):157-65, 1990).

Recently, OSM has also been shown to have potent, pro-inflammatory properties in mice in vivo (Modur V. et al. J. Clin Invest. 100:158-168, 1997) and demonstrates potent synergy with IL-1 to promote articular cartilage degradation in model systems, ex-vivo (Cawston T. Biochemical & Biophysical Research Communications. 215(1):377-85, 1995).

OSM induces a prolonged increase in P-selectin (and E-selectin) in endothelial cells (Yao L. et al. Journal Of Experimental Medicine. 184(1):81-92, 1996), stimulates urokinase-type plasminogen activator activity in human synovial fibroblasts (Hamilton J. et al Biochemical & Biophysical Research Communications. 180(2):652-9, 1991) and is a powerful inducer of IL-6 from endothelial cells(Brown Tj.et al. Journal Of Immunology. 147(7):2175-80, 1991 Oct 1). OSM has recently been measured in RA but not OA synovial fluid (Hui W. et al. Annals Of The Rheumatic Diseases. 56(3):184-187, 1997) and synovium, production of which has been localised to macrophages (1997, Okamoto H et al. Arthritis and Rheumatism 40(6) : 1096-1105) and Cawston et al (1998, Arthritis and Rheumatism, 41(10) 1760-1771). To-date further experiments in this field have been speculative based on the similarity of the IL-6 subfamily members (Carroll G. et al Inflamm. Res. 47 (1998) 1-7).

The present inventors have discovered that OSM has the ability to induce TNFα secretion in macrophages. Contrary to recent data suggesting that OSM upregulates production of tissue inhibitor of metalloproteinase - 1 (TIMP-1) (Nemoto et al 1996, A&R 39(4), 560-566), which complexes with and inactivates MMP-1 and would therefore be expected to decrease collagen release, the inventors discovery that OSM induces TNFα secretion suggested to them that OSM may actually play a role in mediating cartilage destruction. Based on this discovery, the present inventors have demonstrated that therapeutic administration of a neutralising anti-OSM antibody without inhibition of other IL-6 family members can alone ameliorate collagen-induced arthritis in a mouse model. Synergy of OSM with TNFα to promote collagen release from cartilage has subsequently been shown by T. Cawston et al (1998, Arthritis and Rheumatism, 41(10) 1760-1771).

According to the present invention there is therefore provided the use of an antagonist of OSM in the manufacture of a medicament for the treatment or prophylaxis of an inflammatory arthropathy or inflammatory disorder. A particular use of an antagonist of OSM is in the

manufacture of a medicament to prevent or reduce collagen release from cartilage. The invention further provides a method for the treatment or prophylaxis of an inflammatory arthropathy or inflammatory disorder comprising administering an effective amount of an antagonist of OSM to a patient suffering from such a disorder.

The antagonist may function by blocking OSM from interaction with the OSM receptor gp130, or the other OSM receptors, OSMrβ chain or LIFr, or by blocking formation of heterodimers of these proteins, and as such prevent OSM binding and signalling thereby reducing synthesis of pro-inflammatory cytokines and/or MMPs. The antagonist according to the invention may therefore be a ligand for either OSM or one or more of the OSM receptors (gp130, OSMrβ or LIFr) or an agent capable of interfering with these interactions in a manner which affects OSM biological activity. Hereinafter reference to an antagonist to OSM can be taken to mean either an antagonist to OSM itself or to one of its receptors.

The present inventors have also demonstrated that in rheumatoid arthritis synovial vascular endothelium, P and E-selectin co-localise with gp130, the signalling element of type I and II OSM receptors. Without wishing to be bound by theory this indicates that OSM, produced by synovial macrophages might prime RA vascular endothelium to facilitate leucocyte recruitment via upregulation of P and E-selectin. The finding that ligation of L-selectin by either specific antibody or fucoidan (L-selectin agonist) drives human mononuclear cells to secrete OSM may be highly significant in terms of amplification of the inflammatory response, by providing an additional local source of OSM to drive TNFα and P and E-selectin.

Amino acid residues which are important for OSM's interaction with gp130 have been identified. From the published amino acid sequence of OSM (Malik et al., 1989, Mol. Cell Biol., 9(7), 2847-53, DNA sequence entry M27288 in EMBL database, protein sequence entry P13725 in Swissprot) these are G120, Q16 and Q20; N123 and N124 may also play a part (see SEQ ID 12 and below). The first 25 residues are a signal peptide, and the mature protein begins at the sequence AAIGS (SEQ ID 13). The sequence is numbered from the first amino acid of the mature protein as shown.

SEQ ID 12

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                          1  5           15          25          35
MGVLLTQRTL  LSLVLALLFP  SMASMAAIGS  CSKEYRVLLG  QLQKQTDLMQ  DTSRLLDPYI
        45          55          65          75          85          95
RIQGLDVPKL  REHCRERPGA  FPSEETLRGL  GRRGFLQTLN  ATLGCVLHRL  ADLEQRLPKA
       105         115         125         135         145         155
QDLERSGLNI  EDLEKLQMAR  PNILGLRNNI  YCMAQLLDNS  DTAEPTKAGR  GASQPPTPTP
       165         175         185         195         205         215
ASDAFQRKLE  GCRFLHGYHR  FMHSVGRVFS  KWGESPNRSR  RHSPHQALRK  GVRRTRPSRK
       225  227
GKRLMTRGQL  PR
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The invention therefore further provides an antagonist or agent capable of interacting with one or more of these specific residues and or the binding sites they help to define on OSM to alter OSM biological activity.

Inflammatory arthropathies which may be treated according to this invention include rheumatoid arthritis, psoriatic arthritis, juvenile arthritis, inflammatory osteoarthritis and/or reactive arthritis. Inflammatory disorders which may be treated include, amongst others, Crohns disease, ulcerative colitis, gastritis for example gastritis resulting from H. pylori infection, asthma, chronic obstructive pulmonary disease, alzheimer's disease, multiple sclerosis and psoriasis.

Potential antagonists of OSM include small organic molecules, ions which interact specifically with OSM for example a substrate possibly a natural substrate, a cell membrane component, a receptor or a natural ligand, a fragment thereof or a peptide or other proteinaceous molecule, particularly preferred is a non-signalling mutant form of OSM which will block binding of OSM to the OSM receptor, but also modified OSM molecules. Such antagonists may be in the form of DNA encoding the protein or peptide and may be delivered for in vivo expression of said antagonist. Antagonists may be vaccines comprising such protein or peptide molecules or DNA, designed to produce an antagonistic effect towards OSM via induction of antibody responses in vivo targeted towards native OSM. Such antagonists may also include antibodies, antibody-derived reagents or chimaeric molecules. Included in the definition of antagonist is a structural or functional mimetic of any such molecule described above. Also contemplated are nucleic acid molecules such as DNA or RNA aptamers.

Preferred antagonists include small organic molecules. Such compounds may be from any class of compound but will be selected on the basis of their ability to affect the biological activity of OSM through one of the mechanisms described above and will be physiologically acceptable ie non-toxic or demonstrating an acceptable level of toxicity or other side-effects. One class of compounds which may provide useful antagonists are ribonucleosides such as N-(1H-pyrazolo[3,4-d]pyrimidin-4y1) benzamide); Davoll and Kerridge, J. Chem Soc., 2589, 1961)

Other preferred antagonists include antibodies, fragments thereof or artificial constructs comprising antibodies or fragments thereof or artificial constructs designed to mimic the binding of antibodies or fragments thereof. Such constructs are discussed by Dougall et al in Tibtech 12, 372-379) (1994).

Also included in the definition of antibody are recombinant antibodies such as recombinant human antibodies, which may be used. The antibodies may be altered ie they may be "chimaeric" antibodies comprising the variable domains of a donor antibody and the constant domains of a human antibody (as described in WO86/01533) or they may be "humanised" antibodies in which only the CDRs are derived from a different species than the framework of the antibody's variable domains (as disclosed in EP-A-0239400). The complementarity determining regions (CDRs) may be derived from a rodent or primate monoclonal antibody. The framework of the variable domains, and the constant domains, of the altered antibody are usually derived from a human antibody. Such a humanised antibody should not elicit as great an immune response when administered to a human compared to the immune response mounted by a human against a wholly foreign antibody such as one derived from a rodent.

Preferred antagonists include complete antibodies, $F(ab')_2$ fragments, Fab fragments, Fv fragments, ScFv fragments, other fragments, CDR peptides and mimetics. These can be obtained/prepared by those skilled in the art. For example, enzyme digestion can be used to obtain $F(ab')_2$ and Fab fragments (by subjecting an IgG molecule to pepsin or papain cleavage respectively). References to "antibodies" in the following description should be taken to include all of the possibilities mentioned above.

As will be appreciated by those skilled in the art, where specific protein or peptide antagonists are described herein, derivatives of such antagonists can also be used. The term "derivative" includes variants of the antagonists described, having one or more amino acid substitutions, deletions or insertions relative to said antagonists, whilst still having the binding activity described. Preferably these derivatives have substantial amino acid sequence identity with the antagonists specified.

The degree of amino acid sequence identity can be calculated using a program such as "bestfit" (Smith and Waterman, Advances in Applied Mathematics, 482-489 (1981)) to find the best segment of similarity between any two sequences. The alignment is based on maximising the score achieved using a matrix of amino acid similarities, such as that described by Schwarz and Dayhof (1979) Atlas of Protein Sequence and Structure, Dayhof, M.O., Ed pp 353-358.

Preferably the degree of sequence identity is at least 50% and more preferably it is at least 75%. Sequence identities of at least 90% or of at least 95% are most preferred. It will nevertheless be appreciated by the skilled person that high degrees of sequence identity are not necessarily required since various amino acids may often be substituted for other amino acids which have similar properties without substantially altering or adversely affecting certain properties of a protein. These are sometimes referred to as "conservative" amino acid changes. Thus the amino acids glycine, valine, leucine or isoleucine can often be substituted for one another include:- phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); asparate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains) and cysteine and methionine (amino acids having sulphur containing side chains). Thus the term "derivative" can also include a variant of an amino acid sequence comprising one or more such "conservative" changes relative to said sequence.

The present invention also includes fragments of the antagonists of the present invention or of derivatives thereof which still have the binding activity described. Preferred fragments are at least ten amino acids long, but they may be longer (e.g. up to 50 or up to 100 amino acids long).

Further preferred antagonists of OSM for use in the invention are oligonucleotide ligands. Systematic evolution of ligands by exponential enrichment (SELEX) is a protocol in which vast libraries of single stranded oligonucleotides are screened for desired activity against a target protein or other molecule (Tuerk & Gold 1990 Science 249, 505-510, Green *et al*, 1991 Meths. Enzymol. 2 75-86; Gold *et al.*, 1995 Annu. Rev Biochem 64,763-797; Uphof *et al.*, 1996 Curr. Opin. Struct. Biol. 6, 281-288). The product of this screen is a single oligonucleotide sequence termed an aptamer with desired activity, usually high affinity binding, for the target protein. The SELEX procedure is usually initiated with an RNA or DNA library consisting of some 10^{14}-10^{15} random oligonucleotide sequences. In a fully randomised oligonucleotide library, each molecule will exhibit a unique tertiary structure which will be entirely dependent on the nucleotide sequence of that molecule. Thus when screened against a target protein the binding affinity of the oligonucleotide for that protein will be determined by the fit between the shape of the oligonucleotide and epitopes on the target protein. As a consequence of starting from a library of vast diversity it is usual to be able to identify aptamers of sub-nM affinity for the target protein with selectivity for that target protein over other proteins with overall structural homology (Tuerk & Gold 1990 supra, Green *et al*, 1991 supra; Gold *et al.*, 1995 supra; Uphof *et al.*, 1996 supra). Using SELEX methodology RNA or DNA aptamers have been generated to over 100 proteins and small molecules including dopamine (Mannironi *et al*, 1997 Biochemistry 36,9726-9734), substance P (Nieuwlandt *et al*, 1995 biochemisry 34, 5651-5659), human neutrophil elastase (Bless *et al.*, 1997 Current biol. 7, 877-880), Platelet Derived Growth Factor (PDGF) (Green *et al*, 1996 Biochemisry 35, 14413-14424), Vascular Endothelial Growth Factor (VEGF) (Green *et al.*, 1995 Chem Biol. 2, 683-695), thrombin (Bock *et al.*, 1992 Nature 355, 564-66) and L-selectin (O' Connell *et al.*, 1996 PNAS USA 93,5883-5887).

A number of aptamers have been demonstrated to have biological activity, usually receptor antagonism or enzyme inhibition, both *in vitro* and *in vivo*. For example RNA aptamers with high affinity and inhibitory activity to human neutrophil elastase (hNE) were generated by blended SELEX (Bless *et al.*, 1997 supra). Following post-SELEX modification to increase *in vivo* stability the aptamer was tested in a rat model of lung inflammation (Bless *et al.*, 1997supra). In a second example, a 49 nucleotide long DNA aptamer was generated to human L-selectin with nM affinity for the protein (O'Connell *et al.*, 1996 supra). The aptamer exhibits

600-fold selectivity for L-selectin over E-selectin and 10 000-fold selectivity over P-selectin. Intravenous injection of a PEG formulation of the aptamer inhibited trafficking of radiolabelled human PBMC to the lymph nodes, but not to other organs, in a dose dependent manner (Hicke et al., 1996 J. Clin. Invest. 98,2688-2692). In a third example high affinity RNA aptamers have been raised against human VEGF to investigate the role of VEGF in angiogenesis (Jellinek *et al.*, 1994 Biochemistry 33, 10450-10456; Green *et al.*, 1995; Ruckman *et al.*, 1998. J. Biol. Chem 273,20556-20567; Willis *et al.*, 1998 Bioconjug. Chem. 9,573-582). A liposomal formulation of the VEGF aptamer inhibits VEGF induced endothelial cell proliferation in vitro and vescular permeability increase and angiogenesis in vivo (Willis *et al.*, 1998 supra). There is therefore provided an oligonucleotide ligand of OSM or an OSM receptor (OSMR, LIFR, gp130) for use in the invention.

To raise an aptamer for use in the invention as described above OSM or a receptor must first be bound to plates for screening. Iterative rounds of selection and amplification (ie the SELEX procedure) can then be performed in accordance with Fitzwater and Polisky (Meths in Enzymol. 267, 275-301) to generate RNA or DNA aptamers to human OSM. Typically these aptamers are modified RNA aptamers as RNA provides the greatest structural diversity and therefore possibility of generating high affinity molecules. Following the generation of a high affinity aptamer a number of post-SELEX optimisation protocols may be performed to increase aptamer stability, to truncate the aptamer to a core sequence (typically aptamers are 100mers or shorter) that is more amenable to solid phase synthesis thereby reducing the cost of synthesis, and to develop formulations for use in vivo.

In the first of these procedures the aptamer may be truncated to reduce the length of the molecule to a core sequence required for activity. The short core sequence, often between 20 and 40 nucleotides long, will be cheaper and quicker to synthesise and may have increased bioavailability. Information regarding the composition of the core sequence may be obtained from sequence homology comparisons. However, truncation experiments usually involve the synthesis of sequentially shorter aptamers until a minimum sequence required for activity is generated. This usually involves removal of the fixed sequences but there are numerous examples where nucleotides within the fixed sequence have contributed to aptamer affinity (Fitzwater and Polisky, 1996 *supra*; Ruckman J, et al (1998) J. Biol. Chem. 273, 20556-

20567.Green *et al.*, 1995 *supra*). The invention may therefore provides aptamers which are truncated or extended versions of the selected aptamer or one demonstrating greater than 70% homology in sequence to a selected aptamer.

Following truncation a number of base modification experiments may be performed to improve aptamer stability by protection against ribonuclease cleavage. During SELEX it is not possible to include 2' modified purine bases as the T7 polymerase used for *in vitro* transcription will not tolerate this modification. Hence to increase aptamer stability post-SELEX it is usual to replace the purine bases within the aptamer with 2' modified purines. This modification is usually through the use of 2'-0-methyl purines although other modified purines including 2'-amino purines or 2'-fluoro purines may be used (Ruckman *et al.*, 1998 *supra*; Green *et al.*, 1995 *supra*). This has to be done in a sequential manner as this modification, post-SELEX, may also result in a loss of affinity (Green *et al.*, 1995 *supra*).

Following truncation and stabilisation it is possible to generate very large amounts of a short fully modified aptamer that may be synthesised by chemical solid scale synthesis. Many molecules can be added to the 5' end of an aptamer to facilitate aptamer use or to formulate an aptamer for in-vivo delivery. This includes a caged moiety to aid imaging (Hnatowich D. J. (1996) Q. J. Nucl. Med. 40, 202-8.), fluorescein to aid molecular detection (German *et al.*, 1998 Anal. Chem. 70, 4540-5.), a lipid group to aid insertion into a liposome (Willis *et al.*, 1998 supra), or conjugation to a small molecule drug or peptide (Charlton J, et al (1997b) Biochemistry 36, 3018-3026). Generally, the addition of a molecule to the 5' end of an aptamer does not result in a loss of affinity or specificity.

To improve in vivo half-life, aptamers have been modified through the addition of polyethylene glycol (PEG) molecules or through the incorporation into liposomes. In both cases such modification can cause a significant increase in *in vivo* half-life (Willis *et al*, 1998 supra).

In addition to liposomal formulations aptamers have been formulated with both 20K and 40K PEG to increase serum stability *in vivo*. A DNA aptamer has been generated against human L-selectin. To increase *in vivo* stability a 20K PEG ester was coupled to the aptamer through the N-terminal amine moiety. The PEG conjugated aptamer was demonstrated to block L-selectin-

dependent lymphocyte trafficking *in vivo* in SCID mice (Hicke et al., 1996 *supra*). There is therefore provided for use in the invention, a conjugate of an aptamer and a carrier molecule for example PEG. In this embodiment the aptamer and carrier will be linked for example through the N-terminal amine moiety. In addition there is provided a formulation or composition for use in the invention comprising an aptamer and a delivery molecule for example a liposome. In this embodiment there may be no link between the aptamer and the carrier, the aptamer may simply be encapsulated, dispersed or distributed through the carrier.

The aptamers isolated in this study may also be modified for use as diagnostic molecules to detect the presence of human OSM in serum, tissue or other ex vivo samples, or for the detection of human OSM in whole body *in vivo* imaging studies (Charlton J,et al (1997) Chemistry and Biology 4, 809-816.; Hnatowich, 1996 supra). Fluorescein or other fluorescent detection groups can be added to the 5' end of the aptamer molecule to aid in fluorescence detection for applications such as FACS (Fluorescence Activated Cell Sorting) (Davis KA. Et al (1996) Nuc. Acids Res. 24, 702-6.; Charlton *et al.,* 1997supra), ELONA (Enzyme Linked Oligonucleotide Assays) assays (Drolet DW, et al (1996) Nature Biotech. 14, 1021-1025) and other diagnostic applications. The advent of technetium-99m (Tc99m) chelating peptide cages, such as the MAG3 (Fritzberg A. R.,et al J Nucl Med 1986: 27, 111-6) has greatly facilitated the use of a wide range of molecules (Kubo A.et al , (1998) Kaku Igaku 35, 909-28) and macromolecules (Taillefer R.et al , (1995) Eur. J. Nucl. Med . 22, 453-64.), for imaging the presence of the target protein *in vivo* (macromolecules (Pallela V. R., et al (1999) Nucl. Med. 40, 352-60.). Images are visualised with the aid of a γ-camera and have been achieved in a variety of species from mouse to man. Recent modification of the Tc99m chelators has enabled more efficient and stable labeling of molecules under mild conditions (Hnatowich D. J.1998 Nucl Med 39, 56-64.). Methods for radiolabeling single-stranded oligonucleotides have already been developed, the fate of such unmodified labeled oligonucleotides in vivo has been preliminary investigated (Hnatowich, 1996 supra).

It will of course be appreciated that any peptide, protein or nucleic acid based antagonists for use in this invention will preferably be in a purified form ie free from matter associated with such a molecule either in its natural state or as a result of its manufacture, notably the purity is greater than 70% pure but more preferably greater than 80% or 90% pure.

The antagonists of the present invention may be used alone or in combination with immunosuppressive agents such as steroids (prednisone etc.), cyclophosphamide, cyclosporin A or a purine analogue (e.g. methotrexate, 6-mercaptopurine, or the like), or antibodies such as an anti-lymphocyte antibody or more preferably with a tolerance-inducing, anti-autoimmune or anti-inflammatory agent such as a CD4+T cell inhibiting agent e.g. an anti-CD4 antibody (preferably a blocking or non-depleting antibody), an anti-CD8 antibody, an anti-CD23 antibody, a TNF antagonist e.g. an anti-TNF antibody or TNF inhibitor e.g. soluble TNF receptor, or agents such as NSAIDs or other cytokine inhibitors.

Suitable dosages of an antagonist of the present invention will vary, depending upon factors such as the disease or disorder to be treated, the route of administration and the age and weight of the individual to be treated and the nature of the antagonist. Without being bound by any particular dosages, it is believed that for instance for parenteral administration, a daily dosage of from 0.01 to 20 mg/kg of an antibody (or other large molecule) of the present invention (usually present as part of a pharmaceutical composition as indicated above) may be suitable for treating a typical adult. More suitably the dose might be 0.1 to 5 mg/kg, such as 0.1 to 2 mg/kg. A unit dose suitably will be 1-400 mg. Suitable dosages of small organic molecules would be similar and suitable dosages of oligonucleotide ligands would be for example 0.1-10mg/kg.

The invention further provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an antagonist according to the invention and optionally another therapeutic agent as described above. The antagonist, and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously but depending on the nature of the antagonist other routes such as oral, by inhalation, intra-nasal, topical, or intra articular may be more appropriate.

The compositions for parenteral administration will commonly comprise a solution of the antagonist or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These

compositions may be sterilised by conventional, well known sterilisation techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjustment agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody or other antagonist in these formulations can vary widely, for example from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and 50 mg of antagonist. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody or other antagonist according to the invention. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). Suitable formulations for nucleic acid antagonists are discussed above.

The protein antagonists of this invention such as antibodies can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins. Any suitable lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody or other antagonist of this invention sufficient to effectively treat the patient.

The present invention includes within its scope an assay for determining whether or not a particular agent which binds to OSM may be useful in the treatment of an inflammatory disease. The invention therefore comprises an assay for the identification of antagonists of OSM comprising combining OSM with the test agent and determining whether or not the agent is capable of blocking the interaction between OSM and the OSM receptor or affecting OSM biological activity through differential expression of a marker molecule.

To select an antagonist for use in the invention as described above OSM, the key binding residues of OSM as described above presented on a carrier or in a manner in which the binding sites are defined ("OSM binding moiety"), or an OSM receptor must first be obtained. cDNA encoding human OSM may be generated synthetically, based on the EMBL sequence (accession number M27288), cloned into an appropriate expression vehicle and used to transform an appropriate host such as E. Coli. Human OSM protein is then purified from culture medium and bound to plates for screening.

OSM, an OSM binding moiety and/or an OSM receptor may be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. The invention therefore provides an assay for the identification of an antagonist of OSM which comprises contacting OSM with a test agent and measuring for binding. These substrates and ligands may be natural substrates and ligands may be structural or functional mimetics. Such molecules are included in the definition of antagonists of OSM. The method of screening may involve high-throughput. For example, to screen for antagonists, a synthetic reaction mix, cellular compartment, such as a membrane, cell envelope or cell wall, or a preparation of any thereof, may be prepared from a cell that expresses OSM receptor. The preparation is then incubated with labelled OSM in the absence or the presence of a candidate molecule. The ability of the candidate molecule to bind to OSM receptor is reflected in decreased binding of the labelled OSM. Molecules which bind gratuitously, ie, without inducing the functional effects of OSM are most likely to be good antagonists. This assay may be reversed and labelled OSM receptor may be used with unlabelled OSM. A further screen with an ELISA format may be used to identify OSM antagonists where the ability of a candidate molecule to prevent binding of an OSM receptor conjugate such as gp130-Fc fusion protein to

plate-immobilised OSM is measured, in this assay bound gp130-Fc is detected by enzyme-labelled anti-Fc antibody and colourimetric assay.

The functional effects of potential antagonists may be measured, for instance, by determining activity of a reporter system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of OSM or molecules that elicit the same effects on OSM. Reporter systems that may be useful in this regard include but are not limited to colorimetric labelled substrate converted into product, a reporter gene that is responsive to changes in the functional activity of OSM receptor, and binding assays known in the art.

Figures:

Figure 1a: ELISA showing ex-vivo secretion of Oncostatin M by synovial biopsy cultures.

Figure 1b: Spontaneous ex-vivo secretion of Oncostatin M by inflamed but not non-inflamed synovial cultures.

Figure 2: Effect of rhOSM on TNF alpha production by PMA differentiated THP-1 cells.

Figure 3 a&b Synergistic effect of OSM with TNFα to promote collagen release ex-vivo.

Figure 4a Anti-L-selection antibody mediated secretion of OSM.

Figure 4b Fucoidan induced OSM secretion.

Figure 5a-d Photomicrograph demonstrating the staining of RA vascular endothelial using gp130 P and E - selection antibodies.

Figure 6 OSM RNA message in joints from control & CII - arthritic mice.

Figure 7 Arthritic DBA-1 mice treated with goat anti-OSM antibody or control goat IgG. 7a = Clinical scores. 7b = Paw thickness

Figure 8 Histological data comparing joint infiltration and cartilage damage in collagen-arthritic mice.

8a & b: Control mice exhibited extensive joint infiltration by PMNs and mononuclear cells (8a) and surface destruction of articular cartilage, characterised by widespread neutrophil infiltration (8b).

8c & 8d: Representative joints of an anti-OSM treated animal with normal/mild arthritis, demonstrating a markedly reduced level of cellular infiltrate with intact articular cartilage.

Figure 9 HepG2 B6 sPAP and MTS assay for N-(1H-pyrazolo[3,4-d]pyrimidin-4-yl)benzamide showing a concentration-dependent inhibition of OSM-induced sPAP release.

Figure 10 TNFα sPAP and MTS assay for N-(1H-pyrazolo[3,4-d]pyrimidin-4-yl)benzamide showing limited inhibition of TNFα -induced sPAP release from A549 cells.

Figure 11 Antibody inhibition of sPAP production in the HepG2 B6 assay. M2-M4 denote mouse sera from four individual mice; OM5-6.1, OM5-6.10, OM6-10.111 denote experimentally derived hybridoma supernatant.

Figure 12 Competition of wild type and mutant OSM-GST fusion with plate-bound wild type OSM for binding to gp130-Fc in an Elisa.

Figure 13 O.D. plots of three mutant OSM-GSTs showing least activity in driving sPAP production in the HepG2 cells.

The present invention will now be described by way of example only with reference to the accompanying drawings; wherein:

Example 1: Detection of OSM in ex-vivo synovial tissue cultures.

Experiment 1:

Freshly excised synovial tissue from patients diagnosed as having rheumatoid arthritis, osteoarthritis or bunions was mechanically dissected using sterile hypodermic needles to produce approximately 1 mm^3 fragments. These were placed in flat-bottomed 200 µl wells on a 96 well tissue culture plate (Costar) to which was added RPMI 1640 (Sigma) supplemented with 10% heat-inactivated AB^+ male serum (North London Blood Transfusion Centre), 10 mM hepes, 1% sodium pyruvate, 1% non-essential amino acids (all from Sigma), 4 mM L-glutamine (Hyclone), 100 U/ml penicillin + 100 µg/ml streptomycin (Hyclone) (complete human medium, CHM) and incubated at 37°C.

100ul/well samples of culture supernatant were collected on days 0, 2, 5 and 9 frozen at 20°C and then tested for OSM by ELISA. (Quantikine R&D Systems) Data are shown in Fig 1a. Secreted OSM was detected in RA-derived synovial samples, but not from synovium derived from OA or non-arthritic control patients. OSM levels in the RA tissue cultures were maximal around day 5 of incubation, reaching a concentration of approximately 1400 pg/ml and remained greater than

800 pg/ml at day 9.

Experiment 2:

Synovial tissue was washed in PBS and fatty tissue removed. Sterile scissors were used to cut the tissue into small (1-4mm) fragments. This tissue was washed in PBS before use. The tissue was weighed and directly plated out in a 24 or 48 well plate (Costar), 100mg/well. The tissue was cultured at 37°C and 5% CO_2 in 1.5 ml Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated AB+ human serum (Sigma), 2mM L-glutamine (Life Technologies), 200 U/ml penicillin and 200 µg/ml streptomycin (Life Technologies), 480 U/ml nystatin (Sigma), 50µg/ml gentamycin (Life Technologies) and 10 mM Hepes (Sigma), filter-sterilised. At day 3 supernatants were removed and tested for OSM in an ELISA using paired antibodies (R&D Systems).

Synovial tissue cultures from knee biopsy of patients with RA or inflamed OA spontaneously secrete OSM. Following the 3 day incubation period, the mean level of OSM in the culture supernatant from the RA cultures was 246 pg/ml (range 30 to 982, n=12) and from OA cultures was 473 pg/ml (range 44 to 2001, n= 14). OSM is secreted by inflamed but not quiescent synovial tissue (Figure 1b).

Example 2a: Differentiation of THP-1 cells

Human pro-monocytic line THP-1 cells (ECACC) were passaged twice weekly in RPMI supplemented with 10% heat-inactivated FCS, 10 mM hepes, 1% non-essential amino acids (all from Sigma), 4 mM L-glutamine (Hyclone), 100 U/ml penicillin + 100 µg/ml streptomycin (Hyclone) (complete medium, CM) and then PMA (Sigma) was added to washed cells at 1ug/ml and Incubated at 37°C for 30 minutes. Cells were washed x3 in pre-warmed PBS, resuspended in CM and plated out at 1.5 x 10^5 cells/ml in 96-well flat bottomed plates (Costar). Plates were incubated for 48 hours at 37°C, 5% CO_2, then washed with PBS, the media replaced, and incubated for a further 24 hours. Cells were washed x1 in PBS before use.

Example 2b: Preparation of IFN gamma-stimulated blood monocytes

Human buffy coats (North London Blood Transfusion Centre) were diluted 1:3 with PBS, layered onto Lymphoprep (Nycomed UK) and centrifuged at 600xg for 30 minutes at room

temperature. Harvested PBMC were washed 3 times in PBS, counted, and resuspended in 80mls RPMI 1640 / 10% FCS at 5 x 10^6 cells / ml. 20mls were put into each of four 175cm^2 flasks and Incubated overnight at 37°C to allow monocytes to adhere. Non-adherent cells were discarded and adherent cells scraped off after incubation with ice-cold versene at 4°C for 15 minutes. Cells were washed twice in PBS, resuspended in RPMI 1640 + 10% heat inactivated human serum (Sigma) at 6.9 x 10^5 / ml and 250ul cell suspension placed into each well of 96-well flat bottomed plate (Costar). To half the plate was added 100 IU/ml IFN γ (Genzyme), the other half left as control. Plates were incubated overnight at 37°C, 5% CO_2 prior to assay.

Example 2c: Stimulation of TNFα release:

Lyophilised, recombinant human oncostatin M (rhOSM) was purchased from R&D Systems diluted to 10 μg/ml in sterile PBS + 0.1% BSA (Sigma) and aliquots stored at -20°C until use. rhOSM, E. coli-derived LPS or CM were added to triplicate wells of macrophages, monocytes or Thp-1 cells, prepared as above and incubated for 7 hours at 37°C, 5% CO_2. Supernatants were harvested and frozen at -20°C until testing for TNFα protein by ELISA. In assays designed to co-assay for TNFα mRNA, cells were incubated as above for 4 hrs, washed once in PBS and lysed in RNA extraction buffer (RNAzole).

RNA was detected as follows. Total RNA was prepared according to the manufacturer's instructions and stored at -80°C in DEPC-treated water. For RT-PCR, approximately 1ug of RNA was reverse transcribed using oligo dT priming (first strand cDNA synthesis kit, Pharmacia Biotech) and the resulting cDNA subjected to 30 cycles of PCR using the following primers for TNFα (Clontech amplimers): forward- GAGTGACAAGCCTGTAGCCCATGTTGTAGCA, (SEQ ID 1) reverse-GCAATGATCCCAAAGTAGACCTGCCCAGAC (SEQ ID 2). The amplified product (444bp) was separated by agarose gel (2%) electrophoresis and visualized by ethidium bromide staining.

Example 2d: OSM induces TNFα production in human cells of the monocyte lineage

The human pro-monocytic line Thp-1, was induced to differentiate using PMA, washed thoroughly, and incubated with recombinant human OSM as described above. Culture supernatants were removed at 8 hours and assayed for TNFα production by specific ELISA

(TNF Quantikine, R&D Systems) in accordance with manufacturers instructions. OSM induced a dose-related release of TNFα, measurable above 1ng/ml OSM and maximal at 200-500 ng/ml, routinely reaching secreted levels of greater than 2500 pg/ml TNFα. A representative experiment is shown in Fig 2. Expression of TNFα message, measured by RT-PCR as described above was strongly increased in THP-1 cells incubated for 4 hr with 100 *n*g/ml OSM, relative to unstimulated control cells (Fig 2).

Importantly, TNFα induction was not due to contaminating endotoxin as pre-boiling of OSM completely ablated TNFα secretion (data not shown). Also, removal of OSM by immunoprecipitation using specific antibody abolished activity (data not shown). These findings were extended to include human blood monocytes, pre-activated with interferon-γ and human blood macrophages, differentiated in culture for 7 days. Both cell types, when co-incubated for 8 hr with OSM, secreted TNFα, as measured by ELISA. Mean TNFα secretion by monocytes was 1447 pg/ml (range 137-4709 pg/ml; n=4 donors) and 542 pg/ml by macrophages (range 62-1428 pg /ml; n=3 donors).

Example 3a: Cartilage degradation assay

Bovine nasal septum cartilage was held at 4°C overnight after slaughter. 2mm diameter discs were cut from 2 mm slices and washed twice in HBSS. Three discs per well of a 24 well plate (Costar) were incubated at 37°C, 5% CO_2 for 24 hrs in a 600 µl volume of DMEM (Sigma) containing 25 mM HEPES supplemented with 2mM glutamine, 100 µg/ml streptomycin, 100U/ml penicillin and 2.5 µg/ml amphotericin B (cartilage degradation medium, CDM). Cartilage was cultured in quadruplicate wells in either: 600 µl of CDM alone, 2, 10 or 50 ng/ml human recombinant TNFα alone, 10 ng/ml rhOSM alone (R&D systems) or TNFα + OSM and incubated for 7 days at 37°C, 5% CO_2. Supernatants were harvested and replaced with fresh medium containing identical test reagents to day 1. The experiment was continued for a further 7 days and on day 14 all medium was removed and the remaining cartilage digested with 4.5 mg/ml papain (Sigma) in 0.1M phosphate buffer pH 6.5, containing 5mM EDTA and 5mM cysteine hydrochloride, incubating at 65°C for 16 hrs, to determine the remaining hydroxyproline content of the cartilage fragments. The cumulative level of OH-proline released into the medium by day 14 was measured and expressed as the percentage of total released as set out below.

Example 3b: Hydroxyproline assay

Hydroxyproline release was assayed (as a measure of collagen degradation) using a microtitre plate modification of the method in (Bergmann I and loxley R. (1963) Anal. Biochem. 35 1961-1965. Chloramine T (7%) w/v) was diluted 1:4 in acetate citrate buffer (57g sodium acetate, 37.5g tri-sodium citrate, 5.5g citrate acid, 385 ml propan-2-ol per litre water). P-dimethylaminobenzaldehyde (DAB; 20g in 30ml 60% perchloric acid) was diluted 1:3 in propan-2-ol. Specimens were hydrolysed in 6M HCL for 20h at 105°C and the hydrolysate neutralised by drying over NaOH in vacuo using a Savant Speed Vac. The residue was dissolved in water and 40ul sample or standard (hydroxyproline; 5-30ug/ml) added to microtitre plates together with Chloramine-T reagent and then DAB reagent (150ul) after 4 minutes. The plate was heated to 65°C for 35 min, cooled and the absorbance at 560nm determined.

Example 3c: OSM synergises with TNFα to increase MMP1 and collagen release from cartilage explants, ex-vivo.

Bovine nasal cartilage was cultured in quadruplicate wells for 14 days in the presence or absence of OSM or TNFα alone (both from R&D Systems), or in combination, as described above. Culture supernatants were assayed for total collagenase activity on day 7 and for released collagen on day 14. Data in Fig 3b demonstrate that neither OSM nor TNFα alone, used at 10 ng / ml or 50 ng/ml, respectively, induced significant MMP1 secretion. However, the combination of OSM and TNFα used at these concentrations did induce measurable MMP1 release. These findings were accompanied by a striking synergy between OSM and TNFα to increase collagen release from cartilage. Fig 3a shows that OSM alone at 10 ng/ml did not induce collagen release, whereas only the highest concentration of TNFα used (50 ng / ml) had a small, but demonstrable effect (less than 10%). However, the combination of 10 ng / ml OSM with either 50 or 10 ng / ml of TNFα resulted in greater than 80% and 30% collagen release, respectively.

Example 4a: Stimulation of PBMC via L-selectin

Mononuclear cells were isolated from human buffy coats as described above. 5 x 10^5 cells were plated out in 0.5 ml volumes and incubated for 24 hr at 37°C , 5% CO_2 with 60 - 80 kD M. wt. fucoidan (Sigma) anti-L-selectin monoclonal antibodies, LAM1-3 and TQ1 or an isotype

matched control IgG antibody (all from Coulter). Supernatants were assayed for OSM using a specific ELISA assay (Quantikine, R&D Systems), according to the Manufacturer's instructions.

Example 4b: Ligation of L-selectin induces OSM secretion

Mononuclear cells from healthy donors were incubated for 24 hrs with anti-human L-selectin antibodies, (either TQ1 or LAM-1), or an isotype-matched control antibody and culture supernatants assayed by ELISA for OSM. Data in Fig 4a show a dose-dependent induction of OSM using both anti-L-selectin antibodies. Control antibody had a minimal effect. The ability of the L-selectin agonist fucoidan to induce OSM from mononuclear cell cultures was then investigated. Fig. 4b shows that fucoidan was a powerful stimulant of OSM secretion, inducing levels similar to those seen in RA and OA synovial biopsy cultures (Example 1, Experiment 2 Fig 1b,).

Example 5a: Immunohistochemistry

Fresh human tissue samples were frozen in CO_2-cooled liquid hexane and stored in the vapour phase of liquid N_2 until use. 7mm cryostat sections were cut onto 3-Aminopropyltriethoxysilane (APES) (Maddox P. et al J. Clin Path. 40; 1256-1260, 1987) coated glass slides and fixed for 10 minutes at 4°C in 2% paraformaldehyde. Endogenous peroxidase activity was blocked for 20 minutes in 0.05% H_2O_2. Unconjugated, primary monoclonal antibodies were obtained from the following sources: CD62P, CLB, Netherlands; CD62E and gp130 R&D Systems UK. Primary antibodies were applied at optimal dilution for 45 minutes at room temperature. Negative control sections were incubated with an anti-BrdU monoclonal antibody (SIGMA) used at protein concentrations equivalent to test antibodies. A biotinylated secondary antibody, followed by peroxidase labelled ABC (Vector Elite) was used to label the primary antibody. Peroxidase was developed with a DAB (3, 3' Diaminobenzidine) substrate (SIGMA).

Example 5b: Co-distribution of selectins and OSM receptors in RA synovium

The frozen sections of inflamed RA synovial tissue were stained using specific antibodies to gp130, and P and E selectin as described above. Photomicrograph (a) in Fig 5 demonstrates that RA vascular endothelium stained strongly positive for gp130.

Staining of RA synovium for P- and E- selectin expression revealed an identical staining pattern to gp130, restricted to vascular endothelial cells. (Fig 5 b and c respectively). Note in Figure 5c the perivascular mononuclear cell infiltrate associated with E-selectin staining. Staining of serial sections using control primary antibodies was negative on vascular endothelial cells (Fig 5 panels c and d).

Example 6a: Anti-OSM antibody treatment of collagen-induced arthritis.

Collagen induced arthritis was induced in male DBA/1 mice (8-12 weeks old) by immunisation with native bovine type II collagen (CII) as previously described (Plater-zyberk C. Clin. Exp. Immunol 98:442-7 1994 and Plater-zyberk C. Nature Medicine 1: 781-5, 1995). From day 16 post- CII immunisation, mice were monitored daily for signs of joint redness and swelling. From the first appearance of clinical symptoms, mice were examined three times per week and each limb was graded for disease severity using the following visual scores: 0 = normal. 0.5 = arthritis in 2 or more digits, 1 = slight swelling and erythema of paw without digit involvement, 1.5 = same as 1 with involvement of digits, 2 = more pronounced swelling with erythema of paw without digit involvement. 2.5 = same as 2 with digit involvement, 3 = severe swelling with impairment of movement, 3.5 = same as 3 with digit involvement. Paw thickness was measured using calipers (Proctest 2T, Kroeplin Langenmesstechnik).

CII-immunised DBA/1 mice were treated after clinical onset of disease by i.p. injections of 100mg goat anti-mouse OSM antibody (R and D Systems, cat.no. AF-495-NA). Disease progression was assessed as described above. On day 14 post-onset, mice were sacrificed by cervical dislocation and paws collected for histopathological examination.

Example 6b: Histological assessment of arthritic mouse joints

Legs were skinned and knees and paws dissected away. Joints were fixed in 10% buffered formalin for 4 days (knees) or 1 day (paws) and decalcified for 3 days in 25% formic acid, dehydrated and embedded in paraffin wax. Sagittal sections (5-7mm) of the joints were de-waxed and stained with Safranin O, fast green/iron hematoxylin counterstain (as described in Plater-zyberk Nature Medicine above). Synovitis was graded blindly from 0 (no infiltration) to 3 (extensive infiltration and synovial hyperplasia). The degree of loss of Safranin O staining

intensity indicative of cartilage proteoglycan depletion, was scored on a scale from 0 (fully stained cartilage) to 3 (complete depletion and loss of cartilage).

Example 6c: Detection of OSM mRNA in joint tissues of collagen arthritic mice.

Arthritic mice, plus untreated control animals, were sacrificed and both paws and feet removed and snap frozen in liquid nitrogen followed by storage at -80°C. RNA was prepared by grinding each limb in RNAzole using an ultraturrax mechanical homogenizer. Particulate material was allowed to settle, and the supernatant then mixed with 1/10th volume of chloroform and spun to separate the aqueous phase containing RNA. RNA was precipitated using RNAmate (BioChain Institute Inc, San Leandro, California) to remove contaminating proteoglycans. After washing in 75% ethanol, total RNA was dissolved in DEPC-water and reverse transcribed using the Pharmacia first strand cDNA kit and oligo dT priming. PCR reactions were performed using the following primers (Life Technologies custom primers) derived from the mouse OSM sequence(Yoshimura A. et al EMBO Journal 15 1055-1063, 1996): GGGTGTCCTACCAAGGAACA (SEQ ID 3), CTGAGACCTTTCAAGAGGAC SEQ ID 4). After 30 cycles of PCR, reaction products (379bp) were detected using agarose gel electrophoresis. RT-PCR was used to detect OSM mRNA in arthritic mouse paws as described above. Fig 6 shows that levels of OSM-specific PCR product were increased in joints taken from animals with progressively increasing clinical disease scores. By contrast, little or no OSM message was detected in control animals.

Example 6d: Neutralisation of OSM ameliorates collagen-induced arthritis

To directly test the hypothesis that neutralisation might improve clinical symptoms of arthritis, two 100 μg injections of neutralising polyclonal antibody to OSM were administered i.p on days 1 and 3 after the first appearance of clinical arthritis in a group of 6 mice. In parallel, a second group of 6 arthritic mice were treated identically, using non-immune goat IgG instead of anti-OSM. Mice were scored for clinical severity of arthritis, and individual paw swelling measured for a follow-up period of 11 days after the second antibody injection. Mice treated with control goat IgG developed a progressive arthritis, accompanied by an increase in paw swelling.

In marked contrast, mice treated with anti-OSM antibody developed a significantly less severe arthritis in terms of clinical score and paw swelling (Fig 7 a and b). Also, the number of arthritic

paws was significantly reduced in anti-OSM treated compared to control IgG-treated animals, demonstrating that this therapeutic protocol was effective at protecting animals with already established disease from further disease progression. (Data not shown). This experiment was repeated in identical fashion, using 7 mice per group and produced closely matching data (data not shown).

The reduction in clinical severity resulting from treatment with anti-OSM antibody was confirmed by post-mortem histological examination of arthritic paws at day 14 post-disease onset. Histological data comparing joint infiltration and cartilage damage in day 14 collagen-arthritic mice treated with control IgG or anti-OSM antibody are shown in Figure 8. Control IgG treated mice exhibited extensive joint infiltration by PMNs and mononuclear cells (Fig 8a). This was accompanied by surface destruction of the articular cartilage, characterised by widespread neutrophil infiltration (Fig 8b). By contrast, Fig 8c and d show representative joints of an anti-OSM treated animal with minimal arthritis, demonstrating a markedly reduced level of cellular infiltrate, with intact articular cartilage. In addition, joints were scored blindly for histopathological appearance of cartilage and synovium and reported as normal, moderate or severe. A total of 73 individual joints per treatment group were assessed; data are summarised in Table 1. In the animals treated with anti-OSM, 47% of the joints examined were normal or exhibited a mild synovitis, compared to only 6% in the control IgG treated group. Similarly, in anti-OSM treated mice, 58% of the joints examined showed little or no cartilage damage compared to 21% in the control IgG treated group. The joints of the two anti-OSM treated mice with clear signs of joint redness and swelling at day 1 of treatment subsequently showed complete amelioration of arthritis and exhibited neither cellular infiltration nor visible abnormalities to either cartilage or synovium (data not shown).

Table 1: Histological scoring of joints from mice treated with either anti-OSM or control IgG.

Treatment	Normal/Mild		Moderate		Severe	
	cartilage	synovium	cartilage	synovium	cartilage	synovium
anti-OSM	58%	47%	21%	23%	22%	31%
IgG	21%	6%	26%	37%	53%	57%

Total joints examined: 73 joints/treatment

Example 7: Identification of small organic molecule antagonists.

Small organic molecule antagonists of OSM were identified by inhibition of an OSM-induced biological response from a reporter cell line without causing overt cell toxicity. As a control the effect of the compounds on a TNFα- responsive cell-line was also tested.

Example 7a: Expression and purification of human OSM.

A DNA fragment encoding human OSM (hOSM) with the 25 amino acid leader sequence removed was amplified using the Polymerase Chain Reaction (PCR) from an activated leukocyte cDNA library using the synthetic oligonucleotide primers 5'-GCATAGGATCCGCGGCTATAGGCAGCTGCTCG-3' (SEQ ID 5) and 5'-ATCGCGAATTCCTACCGGGGCAGCTGTCCCCT-3', (SEQ ID 6) designed from the EMBL sequence for hOSM (accession number M27288). This PCR product was sub-cloned into pCR2.1 (Invitrogen) to give pCR2.1hOSM.

A SalI restriction endonuclease cleavage site was created within the Factor Xa site in the bacterial expression vector pGEX-3X (Pharmacia) by insertion of ***AC*** for ***TG*** using 'Quickchange' site directed mutagenesis kit (Stratagene) to create the sequence depicted below (SEQ ID 7);

BamHI **EcoRI**

AAA TCG GAT CTG ATC GAA GGT CG*A C*GG ATC CCC GGG AAT TCA TCG

K S D L **I** **E** **G** **R** R I P G N S S (SEQ ID 14)

Factor Xa

Following sequence verification of the OSM insert in pCR2.1hOSM, DNA encoding the mature form of human OSM was PCR amplified from this vector using the forward primer 5'-GATACGATCGTCTCATCGAGCGGCTATAGGCAGCTGC-3' (SEQ ID 8) containing a BsmBI restriction endonuclease site (underlined), and the reverse primer 5'-ATTACATGGAATTCCTATCTCCGGCTCCGGTTCGG-3' (SEQ ID 9) containing an EcoRI site (underlined). This PCR product contains the mature form of human OSM without the leader sequence and without the 31 amino acids from the C-terminus which are removed upon protein maturation. Following PCR, the amplified DNA fragment was purified, digested with

restriction enzymes BsmBI and EcoRI and sub-cloned into the modified pGEX-3X vector (Pharmacia: containing DNA encoding GST) which was restricted with SalI and EcoRI to generate a plasmid designated pGEX 196. Following sequence verification, the plasmid pGEX196 was transformed into *E.coli* BLR-DE3 (Novagen). The transformed cells were cultured in 2xYT+G media (tryptone 16g/l; yeast extract 10g/l; NaCl 5g/l; pH 7.0 with NaOH; 2% glucose) supplemented with 100ug/ml ampicillin.

To prepare purified protein an overnight culture of pGEX 196 in E. coli BLR-DE3 was diluted 1:100 and this culture was grown at 37°C to an A_{600nm} of 0.8. Expression of the GST-hOSM fusion protein was induced by the addition 0.1mM IPTG (Isopropyl-1-thio-β-D-galactopyranoside) and the culture maintained for a further two hours.

GST-hOSM was isolated from the *E.coli* culture by batch purification. A 3 litre bacterial culture was harvested by centrifugation at 3000 rpm and the resulting pellet resuspended in 50ml ice cold PBS (Phosphate Buffered Saline) containing Proteinase inhibitor tablets (Boerhinger). 5ml of lysozyme was added and the cell suspension incubated on ice for 5 minutes. The cells were sonicated at 4°C and 1% Triton X100 and 10mM dithiothreitol was added. The lysate was then end over end mixed at 4°C for 10 minutes, and then centrifuged at 14000g. The supernatant was added to glutathione agarose (Sigma cat no. G4510) and end over end mixed at 4°C for 30 minutes. The suspension was centrifuged lightly, the supernatant aspirated off and the settled agarose was washed twice with ice cold PBS. Elution buffer (20mM glutathione, 100mM Tris pH 8.0, 100mM NaCl; pH 8.0 again) was added and the suspension was incubated on ice for 5 minutes. The supernatant was collected and fractions were analysed by Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining in Coomassie brilliant blue dye, to confirm the integrity of the purified protein.

Proteolytic cleavage optimisation experiments were set up using Factor Xa and thrombin, with thrombin yielding the optimum amount of hOSM as demonstrated by coomassie brilliant blue stained SDS-PAGE analysis. Separation of the GST and OSM products was achieved by ion exchange chromatography and the purified OSM product was verified by N-terminal sequencing and mass spectrometry.

Example 7b: HepG2 B6: OSM-induced sPAP Assay

A HepG2 cell line (ECACC) was stably transfected with six functional STAT3 response elements (REs) upstream of sPAP (secreted placental alkaline phosphatase) cDNA as described below to form HepG2B6. STAT3 (signal transducer and activator of transcription) is an intermediate in the IL-6 cytokine family intercellular signalling cascade. Following dimerisation of cell surface receptors STAT3 is phosphorylated and will then bind to DNA REs in the nucleus and activate DNA downstream, in this construct that DNA is sPAP. Thus this line can be driven to produce sPAP by overnight incubation in Oncostatin M.

A STAT responsive secreted placental alkaline phosphatatse (sPAP) reporter gene was constructed as follows. Initially an oligonucleotide pair containing three copies of a palindromic STAT3 response element (Wegenka U.M et al Mol Cell.Biol, 1993 Vol 13 p276-288 Table 1 on p277) and a 5' Xho1 site was cloned into the unique Sal1 sitc of the plasmid pBluescript tkSPAP to create pllP3-tk-SPAP. A further six copies of a synthetic oligonucleotide encoding the STAT3 response element found in the Fibrinogen β promoter (Dalmon et al, Mol Cell Biol; 1993; 13: 1183-1193 Figure 9 the hβFG sequence including IL6RE Consensus motif and TTG leader without the GAT tail) were then cloned into the Xho1 site of p11P3-tk-SPAP to generate pllx6/llP3-tk-SPAP. Following sequencing to confirm the number of response elements pllx6/llP3-tk-SPAP was digested with Nrul and Xbal to isolate a fragment of DNA containing 9STAT response elements and the tk-SPAP coding sequence. This was subsequently transferred between the Nrul and Xbal sites of the plasmid pcDNA4 (Invitrogen) (replacing the CMV promoter) to create a SPAP gene reporter containing 9 STAT3 responsive elements, and NeoR selectable marker for establishment of the HepG2 cell line.

HepG2 cells (ECACC) were grown in DMEM media supplemented with 2mM L-glutamine, 1% NEAA and 10% HI foetal calf serum at 37°C in an atmosphere of 5% CO_2, 92% humidity. For transfection with the STAT-sPAP reporter, cells were plated at 1% confluence in a 10cm tissue culture dish and transfected with 10ug of the STAT-sPAP reporter vector using a calcium phosphate transfection kit (Invitrogen). Following clonal selection in the presence of 1mg/ml G418 individual cell lines were screened for the ability of IL-6 to cause an increase in the expression of sPAP from the STATsPAP reporter genc.

HepG2B6 cells were plated into 96 well plates to a final concentration of 3 x 10^4 cells per well in 100µl of media (DMEM (Sigma), 10% HI FCS, 1% non-essential amino acids, 2mM Glutamine, 500µg ml^{-1} G418, (all from Life Technologies)). Cells were allowed to equilibrate for 48 hours. Putative anti-OSM solid compounds were made up to a stock dilution of 20mM in DMSO and serially diluted 1:3 in DMSO. This was then further diluted in HepG26B assay media, this is as above media but with 1% heat inactivated FCS, low alkaline phosphatase activity (Life Technologies) substituted for 10% HI FCS. Compounds were diluted 1:3 from a top concentration of 200µM to a final concentration of 0.09µM in a final concentration of 1% DMSO. (That is 200, 66.67, 22.22, 7.41, 2.47, 0.82, 0.27, 0.09 and 0µM). The old media was removed from the wells and replaced with diluted compound also containing 2ng ml^{-1} OSM (R&D Systems), cells were incubated for a further 20 hours. Each dilution was performed in triplicate. 20µl of media was removed from each well and assayed for sPAP activity using pNPP (p-Nitrophenyl phosphate;Sigma), as a substrate. Endogenous alkaline phosphatase is blocked with L-homoarginine. Optical density of substrate is read at 405-650nm. Concentration of compound is plotted against OD as a measure of sPAP produced and can be analysed to determine IC50 values.

Example 7c: A549 cells: TNFα-induced sPAP Assay

This assay used A549 cells that had been stably transfected with a reporter gene, comprising the cytokine responsive region of the E-selectin gene coupled to alkaline phosphatase (Ray et al., Biochem J. 328:707-715, 1997). This transfected cell line can be driven to produce sPAP by overnight incubation with TNFα.

A549 cells were plated into 96 well plates to a final concentration of 5 x 10^4 cells per well in 100 µl of media. Cells were allowed to equilibrate for 24 hours. Putative anti-OSM solid compounds are made up to a stock dilution of 20mM in DMSO and serially diluted 1:3 in DMSO. This was then further diluted in media (DMEM, 1% heat inactivated FCS, low alkaline phosphatase activity, 1% non-essential amino acids, 2mM Glutamine, 500µg ml^{-1} G418, (all from Life Technologies), to give a concentration response of 0.09-200µM in a final concentration of 1% DMSO . The old media was removed from the wells and replaced with diluted compound also containing 3ng ml^{-1} TNFα (R&D Systems), cells were incubated for a further 20 hours. Each

dilution was performed in triplicate. 20µl of media was removed from each well and assayed for sPAP activity using p-Nitrophenyl phosphate (Sigma), as a substrate. Endogenous alkaline phosphatase is blocked with L-homoarginine (Sigma). Optical density of substrate is read at 405-650nm. Concentration of compound is plotted against OD as a measure of sPAP produced and can be analysed to determine IC50 values.

Example 7d: Cell viability assay

Cell viability was measured as the ability of dehydrogenase enzymes in metabolically active cells to reduce a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS to a soluble formazan product that can be directly measured at 490nm.

A solution of 2mg/ml MTS (Promega) containing 0.046µg/ml of phenazine methosulphate (PMS; Sigma) was prepared in Dulbeccos PBS. Following removal of medium for assay of sPAP activity, 20µl/well of MTS/PMS was added. Cells were then incubated for a further 45 minutes. The absorbance at 490nm was then measured using a reference of 630nm.

Example 7e: Antagonists

N-(1H-pyrazolo[3,4-d]pyrimidin-4-yl)benzamide (Davoll and Kerridge, J. Chem. Soc. 2589, 1961) (GW 340442X) produced a concentration-dependent inhibition of OSM-induced sPAP release with an IC_{50} of 0.3µM (Figure 9), but was much less potent at inhibiting TNFα-induced sPAP (approx. IC_{50} value of 92µM) (Figure10). Therefore this compound has greater than 100-fold selectivity for OSM over TNFα.

Example 8a: Generation and testing of anti-human OSM antibodies.

Monoclonal antibodies were raised against human OSM (R+D systems) in mice as follows; SJL female mice (Jackson Inc. Bar Harbor, MA) were immunized with recombinant human OSM (R&D Systems) with either a combination of 1µg of recombinant human OSM antigen emulsified in RIBI adjuvant (RIBI, Hamilton, MT) subcutaneously and 1 µg of antigen in Freund's complete adjuvant intraperitoneally on days 0, 3, 5, and 24 (on day 27, the mouse was given an intraperitoneal injection of 1 µg of antigen in saline); or 1µg of antigen emulsified in

RIBI adjuvant on days 0, 3, 5, 24 and 53 intraperitoneally (on day 54, the mouse was injected with 1.5μg of antigen in saline intraperitoneally).

Twenty four hours after the last immunization, the mice were sacrificed, and splenocytes were harvested and prepared for fusion. The fusion procedure was as described in Su J-L et al : Hybridoma 1998; 17(1): 47-53.). Briefly, splenocytes and myeloma cells P3X63Bcl-2-13 (Kilpatrick KE, et al Hybridoma 1997; 16(4): 387-395) at ratio of 5:1 or 1:1 were fused using polyethylene glycol 1500 (Boehringer Mannheim, Germany). Fused cells were resuspended at 1 x 10^6 cells/ml in hybridoma growth media that is composed of equal volume of RPMI 1640 (Life Technologies, Inc., Gaithesburg, MD) and EXCELL-610 (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 X Origen Hybridoma Cloning Factor (Igen, Gaithersburg, MD), 2mM L-glutamine, and penicillin/streptomycin. Cells were then plated in 24-well microtiter plates (Costar, Cambridge, MA) at 1 ml/well. Twenty four hours later, 1 ml of 2x HAT-selection media; 100 μM hypoxanthine, 0.4μM aminopterin, 16 μM thymine (Life Technologies, Inc.) in hybridoma growth media was added to each well. After 2 weeks of culture at 37°C, 5% CO2, hybridoma supernatants were screened for secretion of anti-OSM antibodies by ELISA. Limiting dilution cloning was performed on selected hybridomas.

Hybridoma supernatants and diluted sera were incubated in 96 well plates containing bound human OSM. Anti-hOSM antibodies were detected by alkaline phosphatase anti-mouse antibodies. Duplicate O.D. values for antibodies giving a positive result are given in Table 2.

Table 2

Hybridoma	**OD 1:10**	**OD 1:100**	**OD 1:1000**
OM5-6.1	1.346 1.329	0.901 0.929	0.302 0.249
OM5-6.10	1.347 1.434	1.017 1.122	0.296 0.352
OM6-10.111	1.77 1.615	1.073 1.557	0.36 .0524
Mouse sera	**OD 1:500**	**OD 1:2500**	**OD 1:5000**
M1	0.006 0.006	0 0.001	0.005 0.003

M2	1.843 1.86	1.086 1.052	0.73 0.794
M3	1.405 1.338	0.445 0.324	0.198 0.217
M4	1.48 1.631	0.537 0.484	0.18 0.18

Three of the supernatants and all but one of the mouse sera gave positive results in the ELISA. Using the ELISA data a crude measure of antibody concentration was determined and the positive antibodies were then titrated against 2ng ml-1 OSM in the HepG2 B6 sPAP assay described in Example 7b. In summary, an antibody was incubated overnight with the cytokine at 4^0C before being incubated with the HepG2 B6 cells. sPAP production was assayed as described in Example7b. Inhibition of sPAP production by the hybridoma supernatants and the mouse sera are shown in Figure 11.

Example 9a: Identification of key binding residues for the receptor on OSM.

The receptor binding sites on hOSM were identified initially by reference to related members of the IL6-family of cytokines. Sites 1 and 3 are thought to be involved in binding to the cytokine specific chain/s of the receptor whilst site 2 is thought to be involved in binding to the common receptor component gp130. Studies of mutations at site 2 in the IL-6 family cytokines leukaemia inhibitory factor (LIF) (Hudson et al (1996) J. Biol Chem 271, 11971-11978), interleukin-6 (IL-6) (Paonessa et al (1995) EMBO J. 14, 1942-1951 and Savino et al (1994) EMBO J. 13 1357-1367) suggest that changes to residues within site 2 can result in altered binding to gp130. In order to investigate the residues of OSM that are important for its interaction with gp130 it was necessary to identify those residues that would be exposed at the surface of OSM in the region of site 2. Using the information from nmr experiments (Hoffman et al (1996) J. Biomol. NMR 7 273-282) and the published structure of LIF (Robinson et al (1994) Cell 77 1101-16) a homology model of OSM was constructed. Residues that occupy surface positions in this model in the site 2 region were selected for mutagenesis. The structure of the complex formed between growth hormone (a homologue of OSM) and its receptor has been determined (De Vos et al (1992) Science 255 306). By superimposing the model of OSM with growth hormone further sites of potential interaction between OSM and gp130 were identified.

Based on these modelling studies, 27 sites were selected for mutation in OSM to investigate its interaction with gp130. See Table 3. At each of these sites an alanine residue was substituted for the wild type residue.

Table 3

Site	Location	Comments
Ser 7	N-terminal region	
Lys 8	N-terminal region	
Glu 9	N-terminal region	
Tyr 10	Helix A	
Arg 11	Helix A	
Leu 13	Helix A	In the current model the exposure of these leucines is borderline. If there is a distortion in the helix below residue 17 the upper part of helix 1 might be rotated and these residues buried
Leu 14	Helix A	
Leu 17	Helix A	
Gly 15	Helix A	

Gln 16	Helix A	Residues 16-22 are an almost continuous run of hydrophilic residues if the underlying structure here is helical then some of these may be buried in the core of the protein and presumably have partnering residues to which they hydrogen bond. Alternatively the helix is distorted in this region and most of these residues are exposed.
Gln 18	Helix A	
Lys 19	Helix A	
Gln 20	Helix A	
Thr 21	Helix A	
Asp 22	Helix A	
Gln 25	Helix A	
Asp 26	Helix A	
Met 113	Helix C	
Pro 116	Helix C	
Asn 117	Helix C	
Leu 119	Helix C	
Gly 120	Helix C	No functionality in side chain but one of a quadruple mutant of human LIF affecting gp130 binding
Arg 122	Helix C	
Asn 123	Helix C	
Asn 124	Helix C	
Tyr 126	Helix C	
Gln 130	Helix C	

Example 9b: Synthesis of mutant OSM-GST fusion molecules

For each of the 27 mutations a pair of mutagenic oligonucleotides was designed. These were approximately 33 bases in length and preferably had a G or C residue at either end. These were annealed to the pGEX (Pharmacia) derived expression containing the 'wild type' OSM DNA (see SEQ ID 12) under the control of a lac (/IPTG inducible) promoter (Pharmacia) and extended

using native Pfu polymerase (Stratagene). The original template DNA was digested with Dpn1 (New England Biolabs) and the newly synthesised plasmid (which was not a substrate for Dpn1) was transformed into the E. coli strain DH5alpha (GibcoBBL/Life Technologies). A small set (typically 4) of colonies was picked, the plasmid DNA was isolated and the DNA sequence was determined. A representative mutant clone for each mutation, along with a similarly constructed wild type were transformed into E coli strain BLR (non DE3: Novagen) for expression of the recombinant proteins. 0.5l cultures were established and induced at an OD550 of approximately 0.5. After 3 hours of induction the cells were pelleted by centrifugation and lysed using a combined method of lysozyme and sonication. Since the recombinant mutant proteins were expressed as fusions with GST, glutathione sepharose columns were used to bind the fusions. The fusion proteins were then eluted from the columns using free glutathione and were then incubated in 10mm DTT for 4 hours at room temperature to remove the glutathione adduct and stored at -80°C.

Example 9c:Effect of point mutations on the ability of OSM-GST to compete with wild type OSM binding to gp130-Fc in an ELISA

Nunc Immunoplates (F6 Maxisorp, Life Technologies) were coated overnight (4°C) with wild type OSM (produced according to Example 7a); 50µl/well, 1µg/ml in carbonate/bicarbonate buffer pH 9.4). Plates were washed (x6 in PBS 0.05% tween 20, using Skatron Plate washer), tapped dry and blocked to reduce non-specific binding (200µl/well, 1% BSA/PBS). Following 1h incubation (room temperature on a shaking platform) the plates were tapped dry and wild type (wt) or mutant OSM-GST from Example 9b added (50µl/well, 20-0.002 µg/ml, titrated in 1%BSA/PBS). As a positive control polyclonal anti-human OSM antibody (R&D Systems) was also tested (20-0.02µg/ml). A complex of gp130-Fc (Produced as below 300ng/ml) and anti-human IgG alkaline phosphatase conjugate (1:500, Sigma) in 1% BSA/PBS (50µl/well) was added immediately after the agents under test. Following a 5h incubation (room temperature on a shaking platform) the plates were washed (x6) and developed using ELISA Amplification System (Life Technologies) as manufacturer's instructions and the OD measured at 490nm. On each plate the total binding was determined by gp130-Fc/conjugate and OSM in the presence of 1%BSA/PBS, and non-specific binding by gp130-Fc/conjugate in absence of OSM, or conjugate binding to OSM in absence of gp130-Fc.

DNA encoding the extracellular domain of human gp130 was amplified by Polymerase Chain Reaction (PCR) using synthetic oligonucleotide primers, forward primer, 5'CATCGGATCCAAGCTTTACAGTTACTGAGCACAGGACCTCACC SEQ ID 10

BamHI HindIII 5'UTR sequence

ATGTTGACGTTGCAGACTTG

M L T L Q T (SEQ ID 15)

and reverse primer 5' CATCCTCGAGTTTCTCCTTGAGCAAACTTTGG SEQ ID 11

XhoI

designed from the GenBank database sequence (accession number M57230) for human gp130. The forward primer contained BamHI, and HindIII restriction endonuclease sites, and a consensus 5' untranslated sequence followed by DNA sequence complementary to the start of the gp130 coding sequence. The reverse primer contained a XhoI restriction endonuclease site followed by DNA sequence complementary to the 3' end of the extracellular domain of the gp130 coding sequence. This PCR fragment was purified and sub-cloned into pCR2.1 (Invitrogen) to give pCR2.1gp130.

The plasmid pCR2.1gp130 was digested with restriction enzymes BamHI and XhoI and the gp130 fragment was purified and sub-cloned into the BamHI and XhoI endonuclease sites in a plasmid containing a DNA sequence encoding an Fc fragment of human IgG1. The plasmid was then digested with the restriction enzyme HindIII, and the resulting gp130Fc fragment was purified and subcloned into the HindIII site of a baculovirus expression vector, pFastBac1 (Life Technologies), to generate a plasmid designated pBACgpFc.

The fusion protein gp130Fc was expressed in insect cells using the Bac-to-Bac baculovirus expression system (Life Technologies) and was then purified from the cell culture supernatant by protein A affinity column chromatography and verified by coomassie brilliant blue stained SDS-PAGE and by western blot analysis using commercially available anti-gp130 and anti-hIgG antibodies.

Mutant and wt OSM-GST were tested to obtain IC_{50} in 3-6 experiments. The mean OD in the presence of OSM and gp130-Fc in the absence of competing ligand (ie total binding) was 1.157

(range 0.825-1.807) and the non specific binding was less than 0.08. The anti-OSM antibody produced a concentration-dependent inhibition in all assays (74±1% inhibition at 1 μg/ml). The wt OSM-GST competed with plate-bound OSM to give a concentration dependent inhibition (Fig. 12), with an IC50 of 0.139±0.0258μg/ml determined in 6 independent experiments. The potency of mutant OSM-GST at competing with plate-bound wt OSM is summarised in Table 4. Mutations which resulted in a substantial decrease in the ability to compete with wt OSM for gp130 binding were L13A, Q16A, Q20A, G120A, N123A and N124A. Of these, Q20A and Q16A were the weakest: at the maximum concentration tested (10μg/ml) Q20A produced 66±2.3% and Q16A only 15±8% inhibition (Figure 12)

Table 4: Potency of wt and mutant OSM-GST at competing with plate-bound wt OSM for binding to gp130-Fc in the ELISA. IC_{50} values were determined in 3-6 independent experiments.

Mutant	IC50 [μg/ml]				Mean	Std. Error
wild type	0.110	0.120	0.257		0.139	0.026
	0.136	0.070	0.142			
(1) S7A	0.199	0.078	0.121		0.133	0.035
(2) K8A	0.252	0.055	0.106		0.138	0.059
(3) E9A	0.208	0.163	0.097		0.156	0.032
(4) Y10A	0.320	0.180	0.168		0.223	0.049
(5) R11A	0.181	0.255	0.280		0.239	0.030
(6) L13A	2.960	1.990	2.640		2.530	0.285
(7) L14A	0.660	0.470	0.412		0.514	0.075
(8) G15A	0.090	0.203	0.171		0.155	0.034
(9)Q16A	>10	>10	>10		>10	
(10) L17A	2.210	1.900	1.350		1.820	0.251
(11) Q18A	0.320	0.310	0.555		0.395	0.080
(12) K19A	0.047	0.075	0.300	0.040	0.116	0.062
(13) Q20A	4.130	5.570	4.100	6.200	5.000	0.527
(14) T21A	0.168	0.044	0.101		0.084	0.020
(15) D22	0.040	0.080	0.092		0.071	0.016
(16)M113A	0.511	0.199	0.252		0.321	0.096
(17)P116A	0.232	0.169	0.197		0.199	0.018
(18)N117A	0.983	0.756	0.617		0.785	0.107
(19)L119A	0.272	0.266	0.227		0.255	0.014
(20)G120A	3.650	2.680	2.950		3.090	0.289
(21)R122A	0.140	0.220	0.167		0.176	0.024
(22)N123A	4.750	1.570	2.560		2.960	0.940
(23)N124A	1.630	1.950	2.380		1.990	0.217
(24)Y126A	0.386	0.359	0.400		0.382	0.012
(25)Y130A	0.145	0.180	0.094		0.140	0.025
(26)Q25A	0.042	0.036	0.055		0.044	0.006
(27)D26A	0.170	0.280	0.481		0.310	0.091

Example 9d:Effect of point mutations in OSM on production of OSM driven sPAP in a HepG2 B6 *in vitro* Assay

The assay described in Example 7b above was employed. OSM-GST mutants were diluted to a concentration of 100ng ml^{-1} using the known concentration of intact OSM mutants generated in Example 9b. A wild type OSM-GST was included for control purposes. Dilutions were made in HepG2 B6 media with 1% heat inactivated FCS, low alkaline phosphatase activity. Serial 1:3 dilutions were then made. (100; 33.33; 11.11; 3.7; 1.23; 0.4 ng ml^{-1}). 3 x 10^4 HepG2 B6 were dispensed into individual well of a 96 well plate in 100µl of media. Cells were allowed to equilibrate for 48 hours. Media was then removed and replaced with 100µl of diluted OSM-GST mutant. Cells were incubated for a further 20 hours. Each dilution was performed in triplicate. 20 µl of media was removed and assayed for sPAP using pNPP as a substrate. Endogenous ALP was blocked with L-homoarginine. O.D. was read at 405-650nm. The experiment was repeated twice.

Most of the mutants could drive sPAP release in a similar manner to the wild type. Three mutants produced very low levels of sPAP. EC_{50}s were not obtained from these mutants. (Figure 13) shows the O.D. plots obtained from mutants 9 (Q16A), 13 (Q20A) and 20 (G120A), which were less effective at driving sPAP production. The wt OSM-GST is shown for comparison. These data were used to calculate EC_{50} values. Actual EC_{50}s for each mutant and expressed as a percentage of the wild type are shown in Table 5.

Table 5

Mutant	Expt 1 %	Expt 1 EC_{50} ng ml^{-1}	Expt 2 %	Expt 2 EC_{50} ml^{-1}	Expt 3 %	Expt 3 EC_{50} ng ml^{-1}	Mean %	'Potency'
WT	100	24	100	32			100	
1 S7A	50	12	69	22.2			59.5	MORE
2 K8A	66	16	38	12.5			52	MORE
3 E9A	98	23.6	27	8.9			62.5	MORE
4 Y10A	134	32.4	256	82			195	LESS

5 R11A	118	28.6	86	27.7			102	EQUAL
6 L13A	269	65.7	171	54.9			220	LESS
7 L14A	81	19.4	77	24.9			79	MORE
8 G15A	87	21	55	17.8			71	MORE
9 Q16A		NC		NC				NONE
10 L17A	301	72.7	174	56			237.5	LESS
11 Q18A	84	20.2	68	21.7			76	MORE
12 K19A	98	23.6	37	11.9			67.5	MORE
13 Q20A		NC		NC				NONE
14 T21A	71	17	33	10.5			52	MORE
15 D22	152	36.7	50	16			101	EQUAL
16 M113A	106	25.6	78	25			92	EQUAL
17 P116A	104	25	47	15			75.5	MORE
18 N117A	241	58	132	42.5			186.5	LESS
19 L119A	115	27.8	72	23			93.5	EQUAL
20 G120A		NC		NC				NONE
21 R122A	135	32.4	43	13.8	124	47.3	101	EQUAL
22 N123A	157	37.9	154	49.7			155.5	LESS
23 N124A	125	30.2	113	36.2			119	EQUAL
24 Y126A	386	93	32	10.3	106.5	40.8	175	LESS
25 Q130A	52	12.5	26	8.2			39	MORE
26 Q25A	55	13.3	41	13			48	MORE
27 D26A	81	19.5	79	25.5			80	EQUAL

EC_{50} values expressed as a percentage of Wild Type EC_{50} and actual EC_{50} values are shown. < 80% More potent; 80-120% Equal potency; > 120% Less potent than wild type.
NC – not calculated

Examination of this table shows three of those mutants which are substantially different from wt in the ELISA are also less potent in the sPAP assay, 6 – L13A; 10 – L17A; 22 – N123A and the fourth, 23 – N124A fall just into the equally potent grade by the arbitary scoring system. Thus both assay types show good concordance. Several of the mutants were less 'potent' than wild type in driving sPAP production but there was variation between the two experiments, except in those mutants (Q16A, Q20A, G120A) that didn't drive sPAP at all.The assay results indicate that

G120A, Q16A and Q20A effect binding of OSM to gp130. N123A and N124A also appear to have some effect on interactions with gp130.

Example 10: Role of OSM in gastritis.

H.pylori is a Gram negative spiral shaped bacterium that has been implicated in causing gastritis, peptic ulcer disease and gastric cancer. H.pylori Cag+ strains have a higher incidence with ulcers than H.pylori Cag- strains. H.pylori strains (more pathogenic Cag+, and Cag-) were co-cultured in-vitro with gastric epithelial cell line KATO III (ECACC) to investigate the host response to H.pylori infection by differential gene expression analysis. mRNA was isolated at time points : 45mins, 3 hours and 24hours, derived radioactive probes were hybridised to high density cDNA gene arrays (containing approximately 136 human genes including cytokines, cytokine receptors and adhesion molecules). Analysis of the gene expression profiles obtained revealed induction/repression of numerous genes in response to the H.pylori strains. Oncostatin M was found to be induced in cells exposed to the highly pathogenic strain of H.pylori (Cag+) compared to cells exposed to the weakly pathogenic H. pylori (Cag-) or un-treated control cells.

Claims

1. The use of an antagonist to OSM or an OSM receptor, for the manufacture of a medicament for the treatment of an inflammatory arthropathy or an inflammatory disorder.

2. The use according to claim 1 wherein the antagonist is an antagonist to human OSM.

3. The use according to claim 2 wherein the antagonist interacts with one or more of the residues G120,Q16,Q20, N123 or N124 of human OSM.

4. The use according to claim 1 wherein the antagonist is an antagonist of the OSM receptor gp130.

5. The use according to any of the preceding claims wherein the antagonist is a small organic molecule.

6. The use according to any of claims 1-4 wherein the antagonist is an antibody.

7. The use according to claim 6 wherein the antibody is humanised or chimaerised.

8. The use according to any of the preceding claims wherein the medicament is used to prevent or reduce collagen release from cartilage.

9. The use according to any of the preceding claims for the treatment of rheumatoid arthritis.

10. A pharmaceutical composition comprising a unit dose of at least 1mg, of an antagonist to OSM, and a pharmaceutically acceptable carrier.

11. A use or pharmaceutical composition according to any one of the preceding claims, wherein the antagonist is in combination with an immunosuppresive, tolerance inducing, or anti-inflammatory agent.

12. A use or pharmaceutical composition according to claim 11 wherein the antagonist is in

combination with a CD4+T cell inhibiting agent, an anti-CD23 antibody or a TNF antagonist.

13. An assay for the identification of an antagonist of OSM comprising combining OSM or an OSM binding moiety and an OSM receptor or receptor conjugate with a test agent and monitoring for blocking of the interaction between OSM or the OSM binding moiety and the OSM receptor or receptor conjugate.

14. An assay according to claim 13 wherein the receptor conjugate is a gp130-Fc fusion protein.

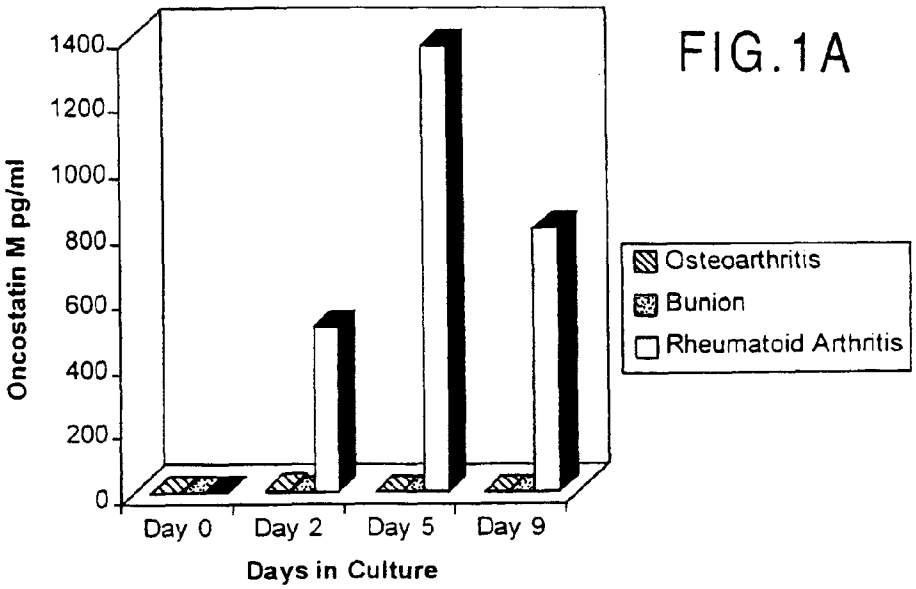


FIG.1A

FIG.1B Spontaneous OSM release from synovial tissue

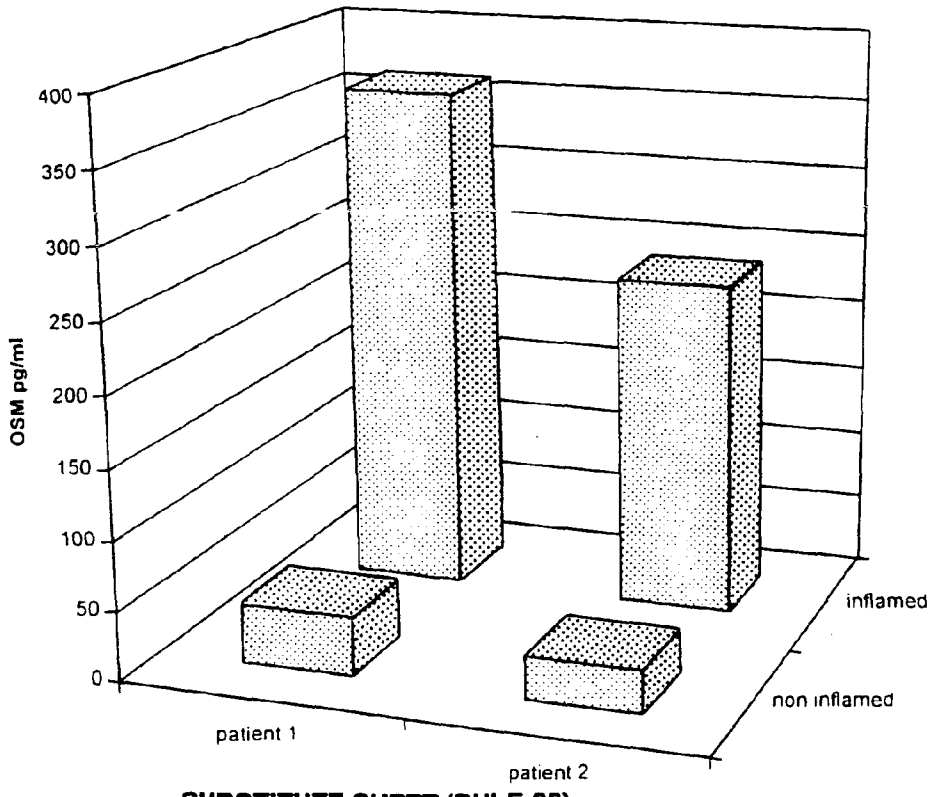


FIG.2

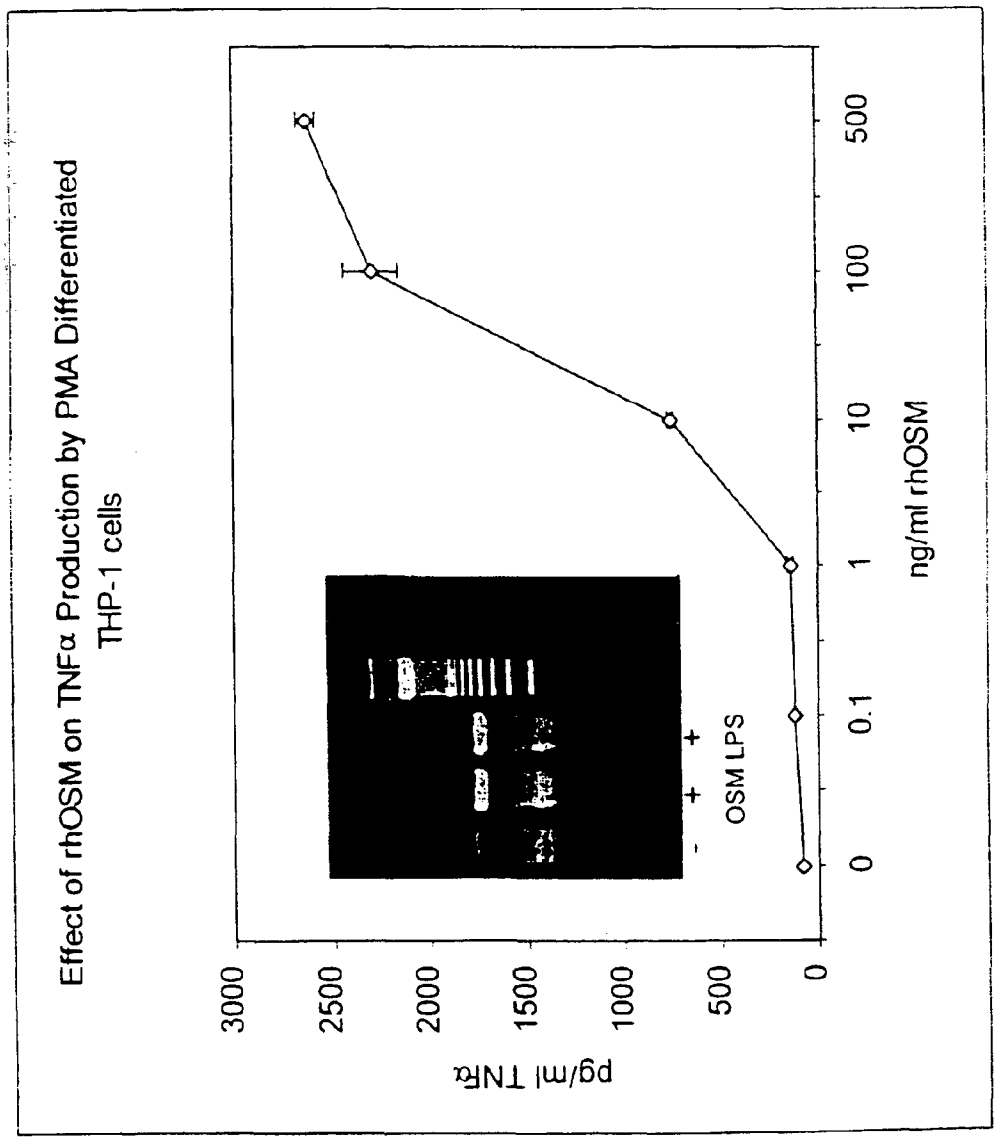

Effect of rhOSM on TNFα Production by PMA Differentiated THP-1 cells
pg/ml TNFα
3000
2500
2000
1500
1000
500
0
0
0.1
1
10
100
500
ng/ml rhOSM
OSM
LPS
+
+
-

FIG.3A

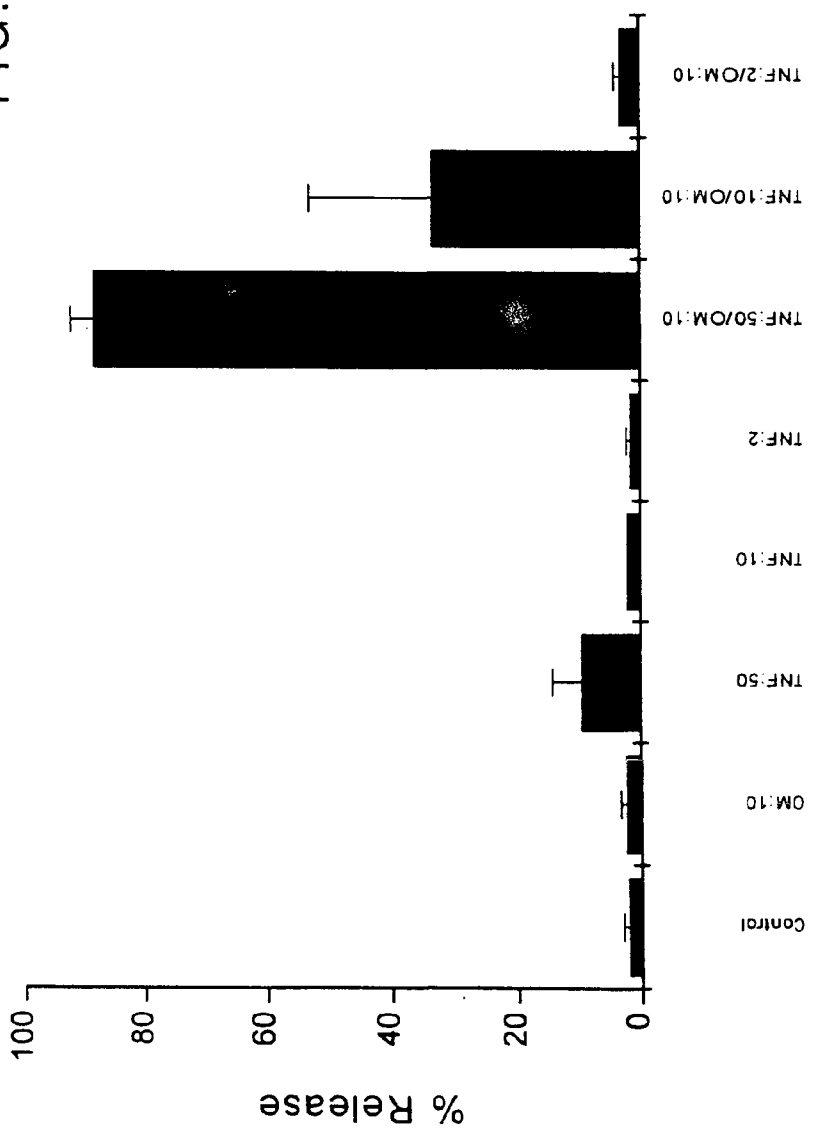
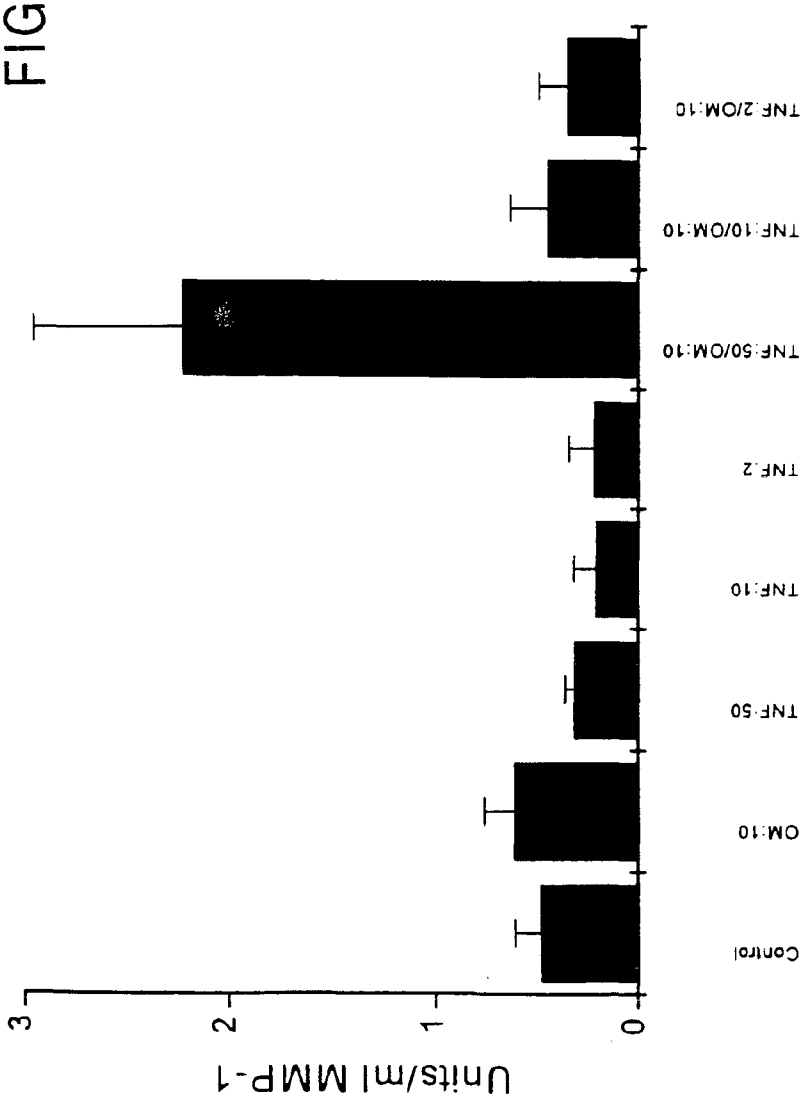

Control
OM:10
TNF:50
TNF:10
TNF:2
TNF:50/OM:10
TNF:10/OM:10
TNF:2/OM:10
0
20
40
60
80
100
% Release

FIG.3B


Units/ml MMP-1
0
1
2
3
Control
OM:10
TNF:50
TNF:10
TNF:2
TNF:50/OM:10
TNF:10/OM:10
TNF:2/OM:10

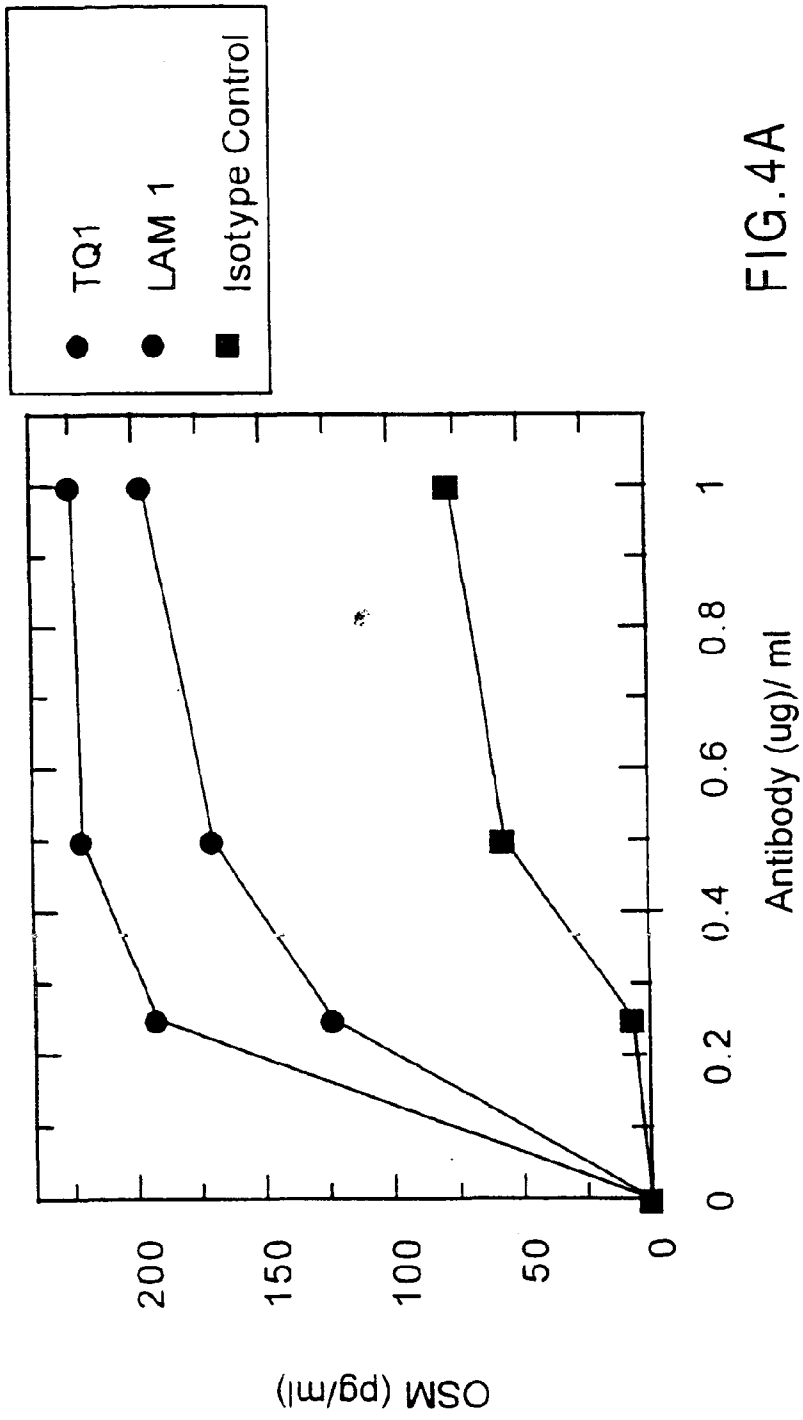

TQ1
LAM 1
Isotype Control
OSM (pg/ml)
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1
Antibody (ug)/ ml

FIG.4A

FIG.4B

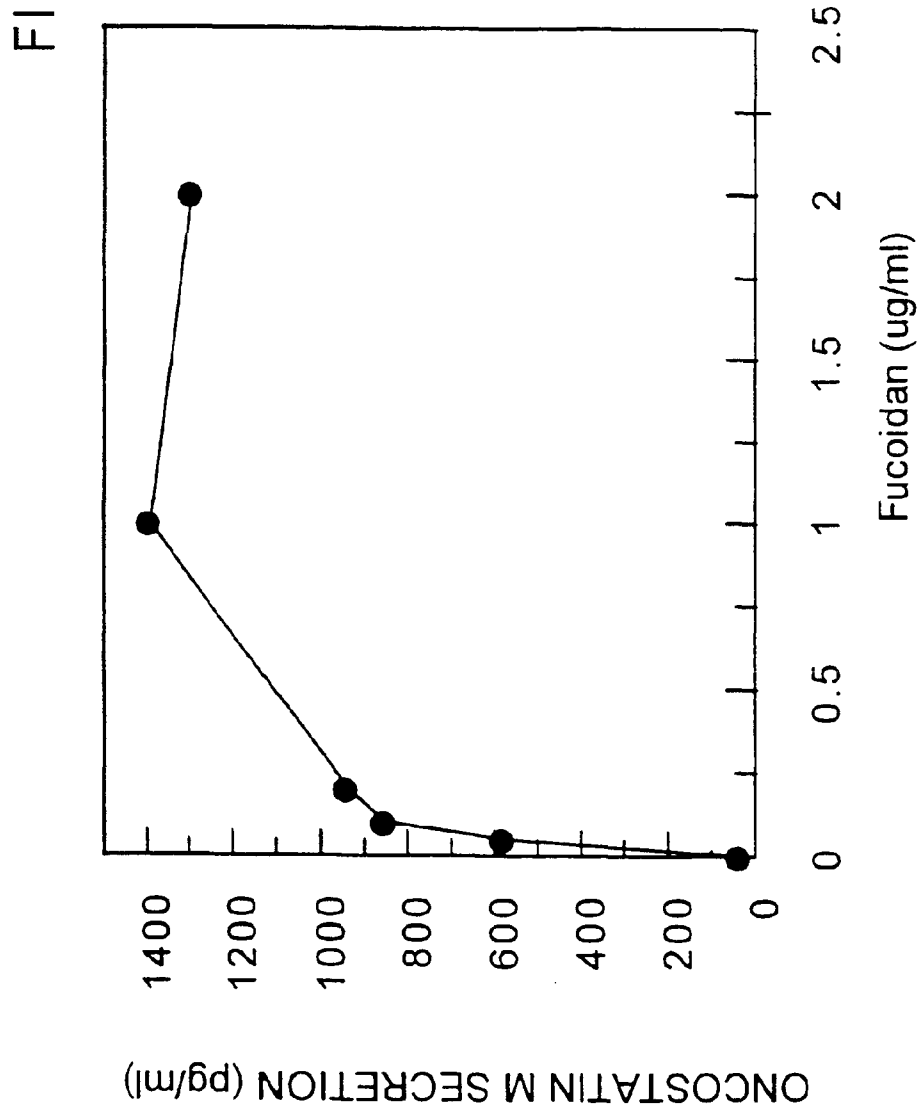

ONCOSTATIN M SECRETION (pg/ml)
1400
1200
1000
800
600
400
200
0
0
0.5
1
1.5
2
2.5
Fucoidan (ug/ml)

FIG.5a

Rheumatoid Arthritis
CD130 localised to endothelial cells of synovial vessels.

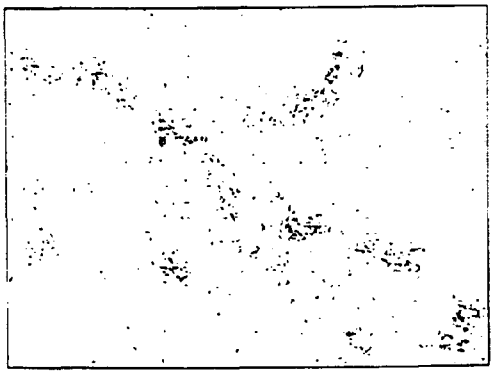


FIG.5b

Rheumatoid Arthritis
Serial section of Figure 5a showing localisation of CD62P to the endothelial cells of the same vessels.

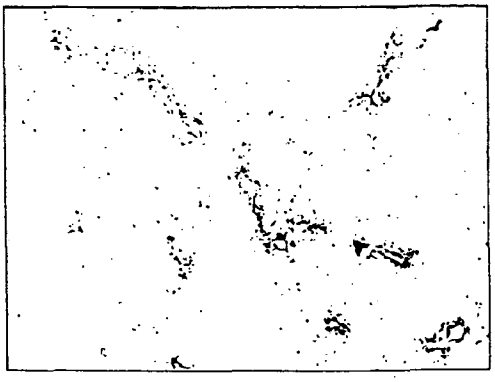


FIG.5c

Rheumatoid Arthritis
Negative Control (slightly higher power) showed no positivity.

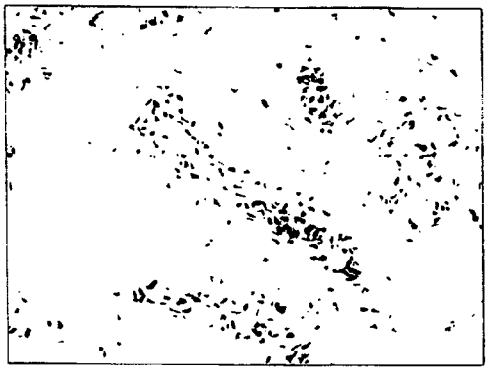


FIG.5d

Rheumatoid Arthritis
CD62E localised to endothelial cells. Small positive vessels can be seen within the lymphoid aggregate at the top of the picture.

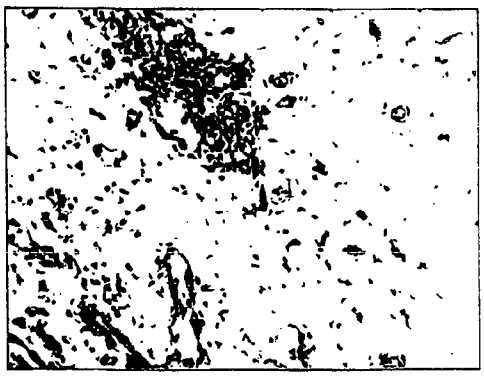


FIG.6

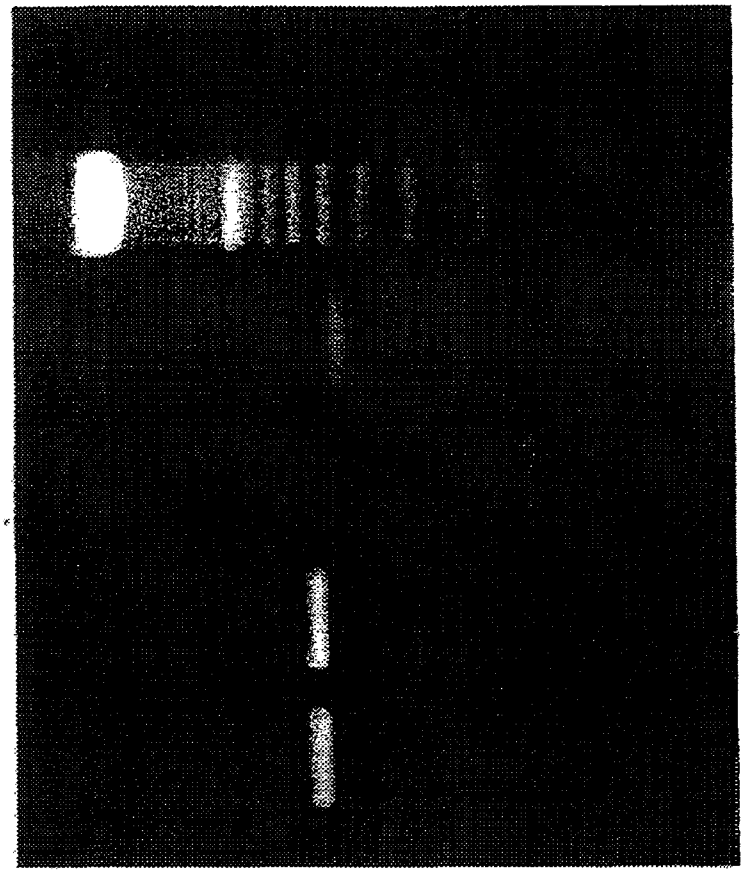


Figure 7.

Arthritic DBA/1 mice treated on day 1 and day 3 of clinical symptoms with goat anti-OSM Ab or control goat IgG (100 µg/injection).

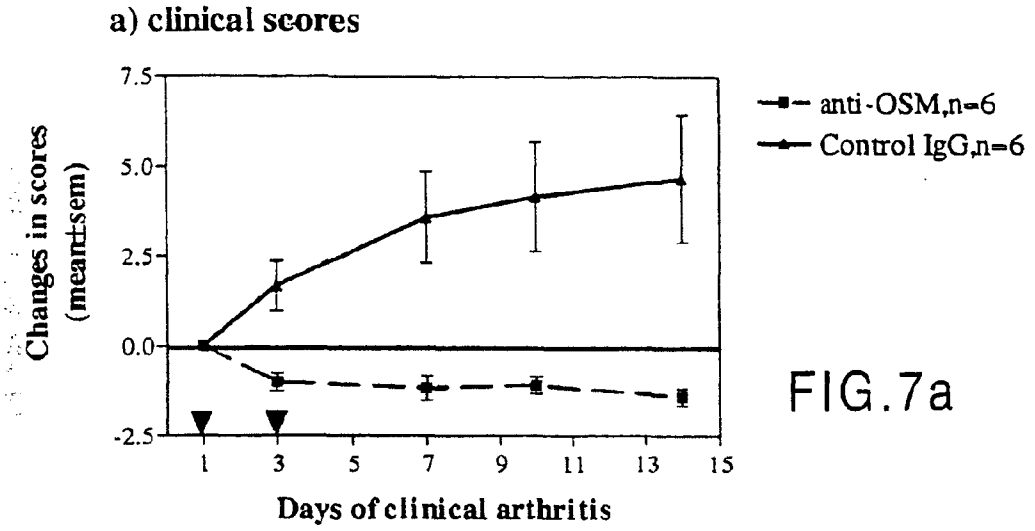


FIG.7a

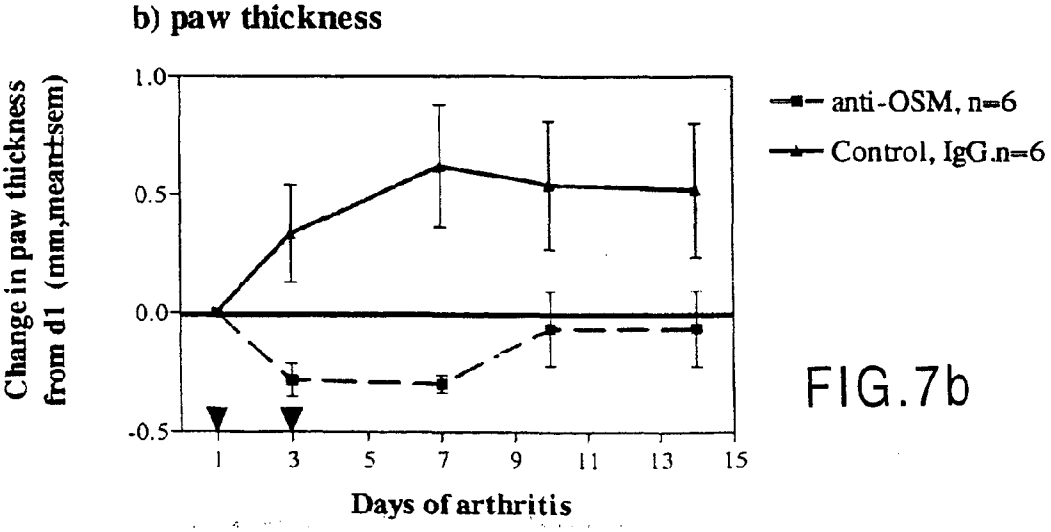


FIG.7b

FIG.8a

FIG.8b

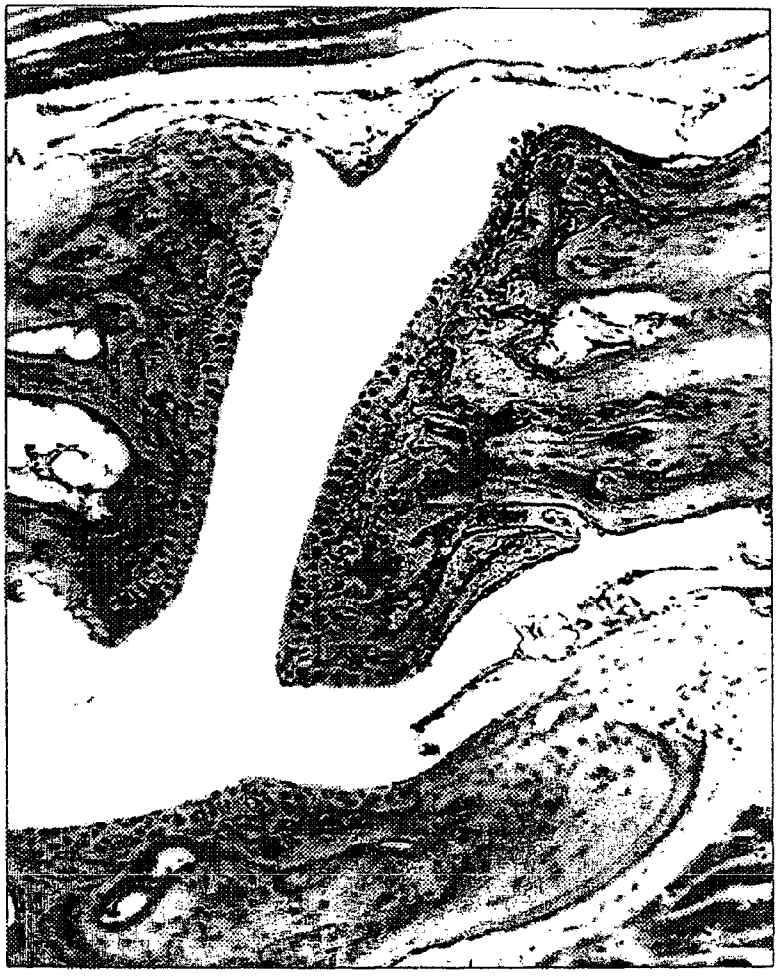


FIG.8c

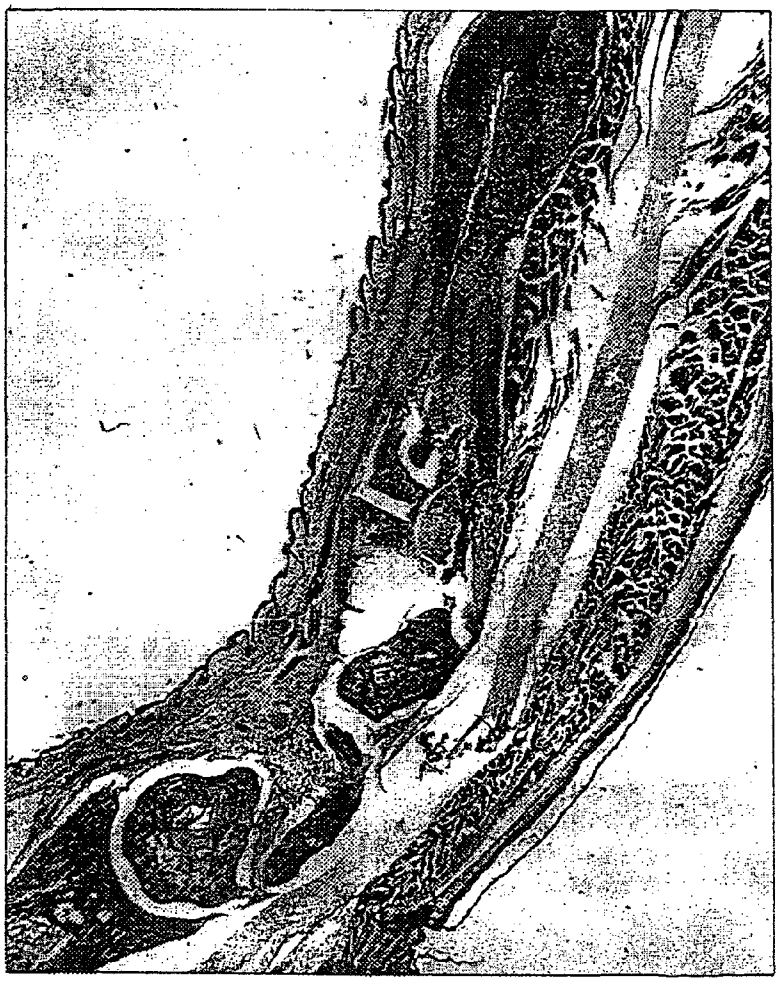


FIG.8d

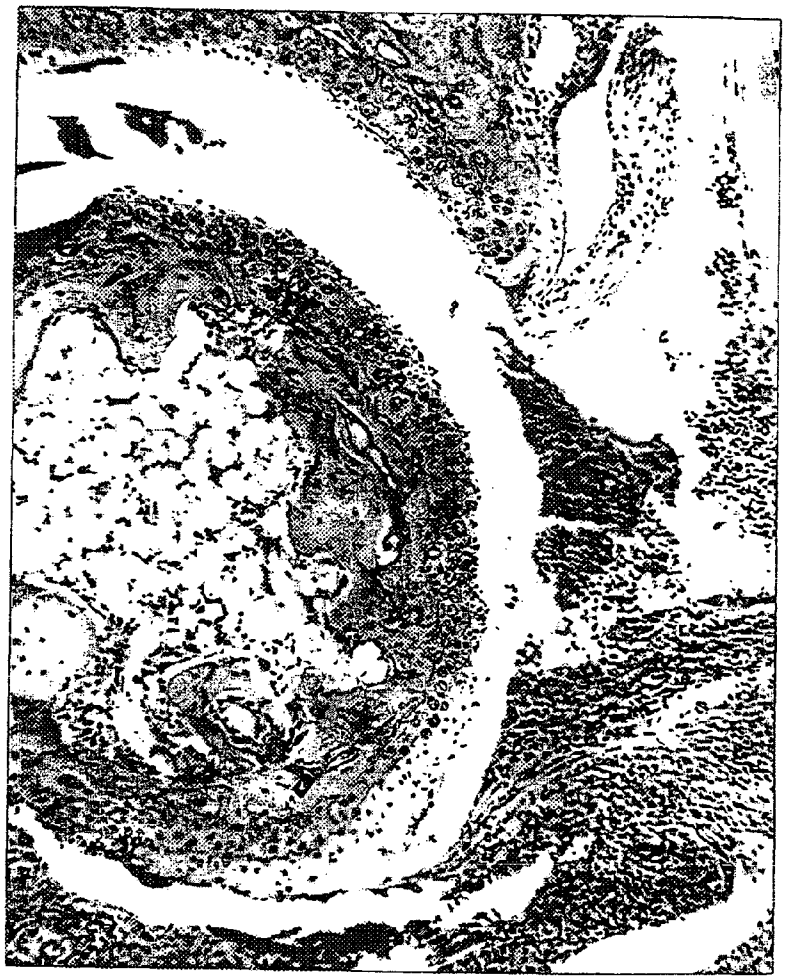
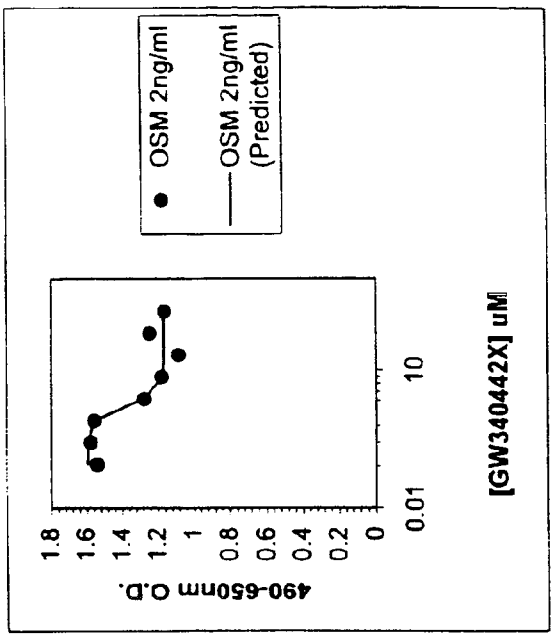
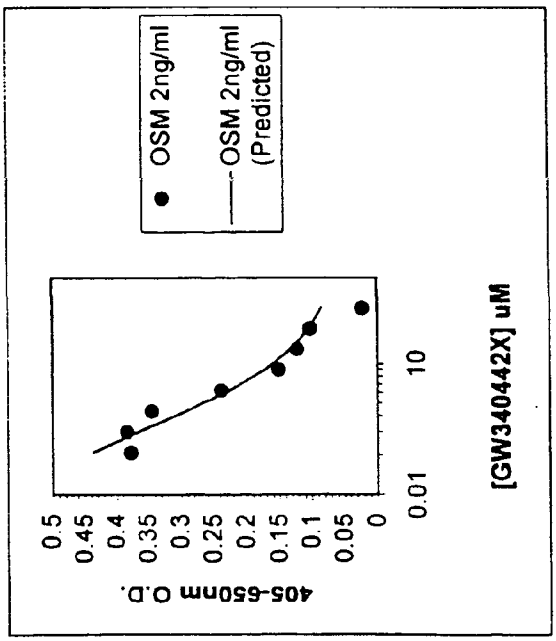


FIG.9



Parameter	Value	SE	Lower CL	Upper CL
a (OSM 2n	1.164684	0.033123	1.07954	1.249829
c (OSM 2ng	1.752591	0.516752	0.821322	3.739794
d (OSM 2n	1.59442	0.038108	1.496461	1.692379
b (OSM 2n	-3.003396	1.935519	-7.978799	1.972006

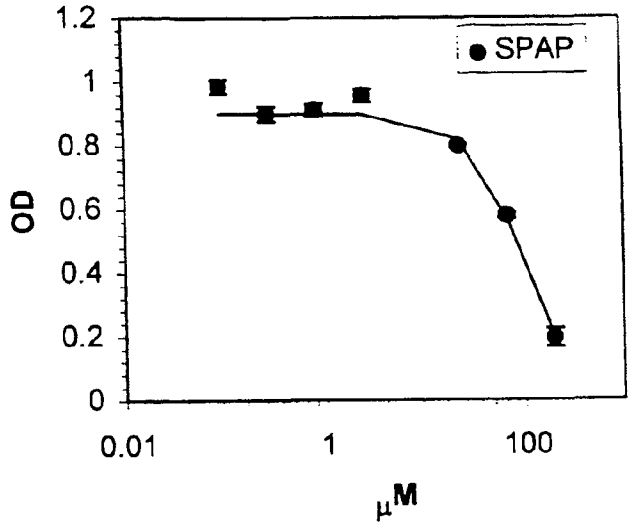
MTS Assay



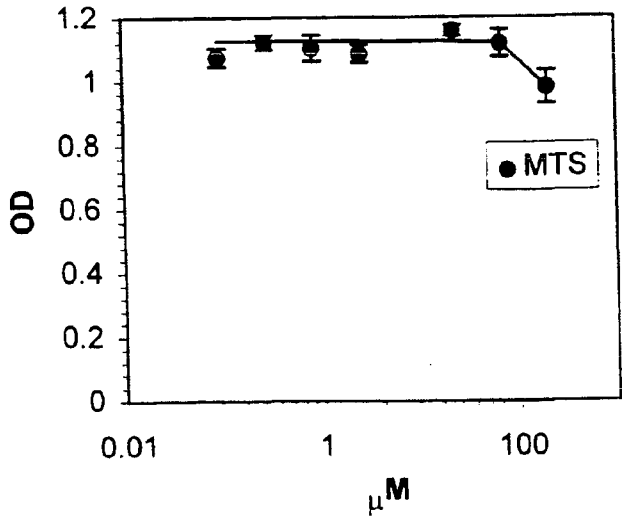
Parameter	Value	SE	Lower CL	Upper CL
a (OSM 2n	0.055	0	0.055	0.055
c (OSM 2ng	0.315071	0.155605	0.09[illegible]098	1.05496
d (OSM 2n	0.654037	0.043523	0.5[illegible]754	0.760534
b (OSM 2n	-0.452621	0.08358	-0.65[illegible]134	-0.248109

sPAP Assay

FIG.10

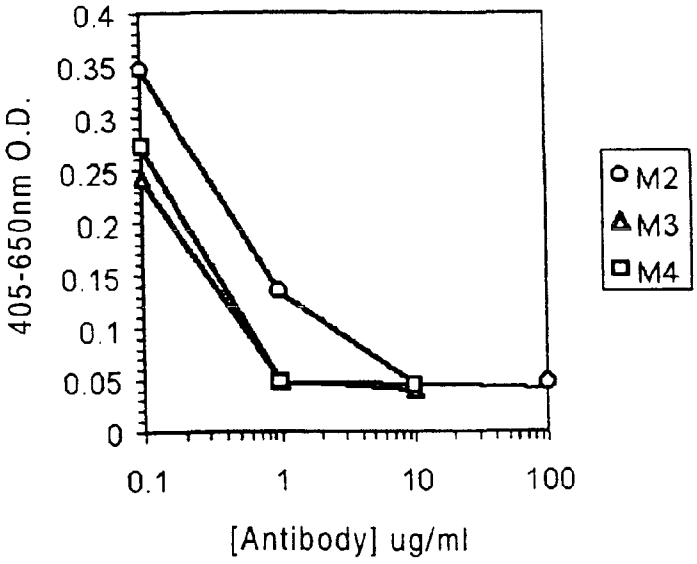


GW340442X



GW340442X

FIG.11


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
405-650nm O.D.
0.1
1
10
100
[Antibody] ug/ml
M2
M3
M4

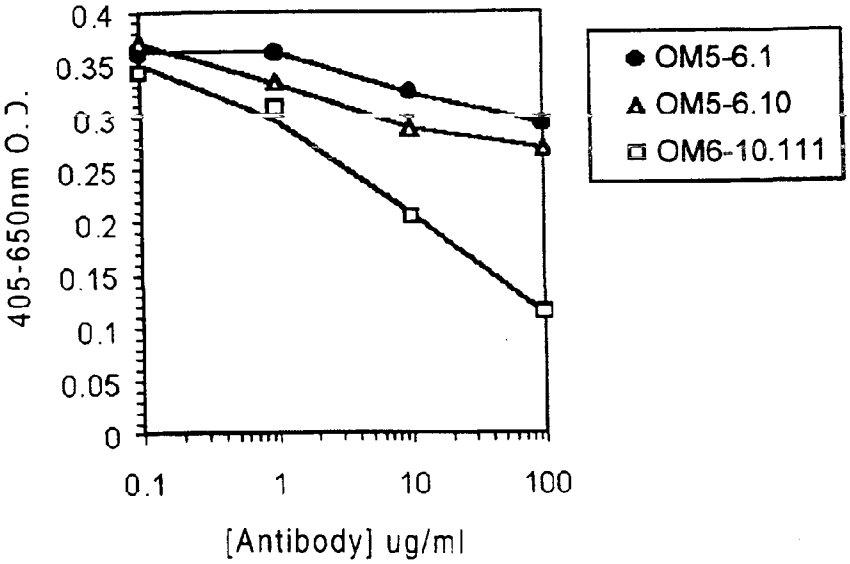

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
405-650nm O.D.
0.1
1
10
100
[Antibody] ug/ml
OM5-6.1
OM5-6.10
OM6-10.111

FIG.12

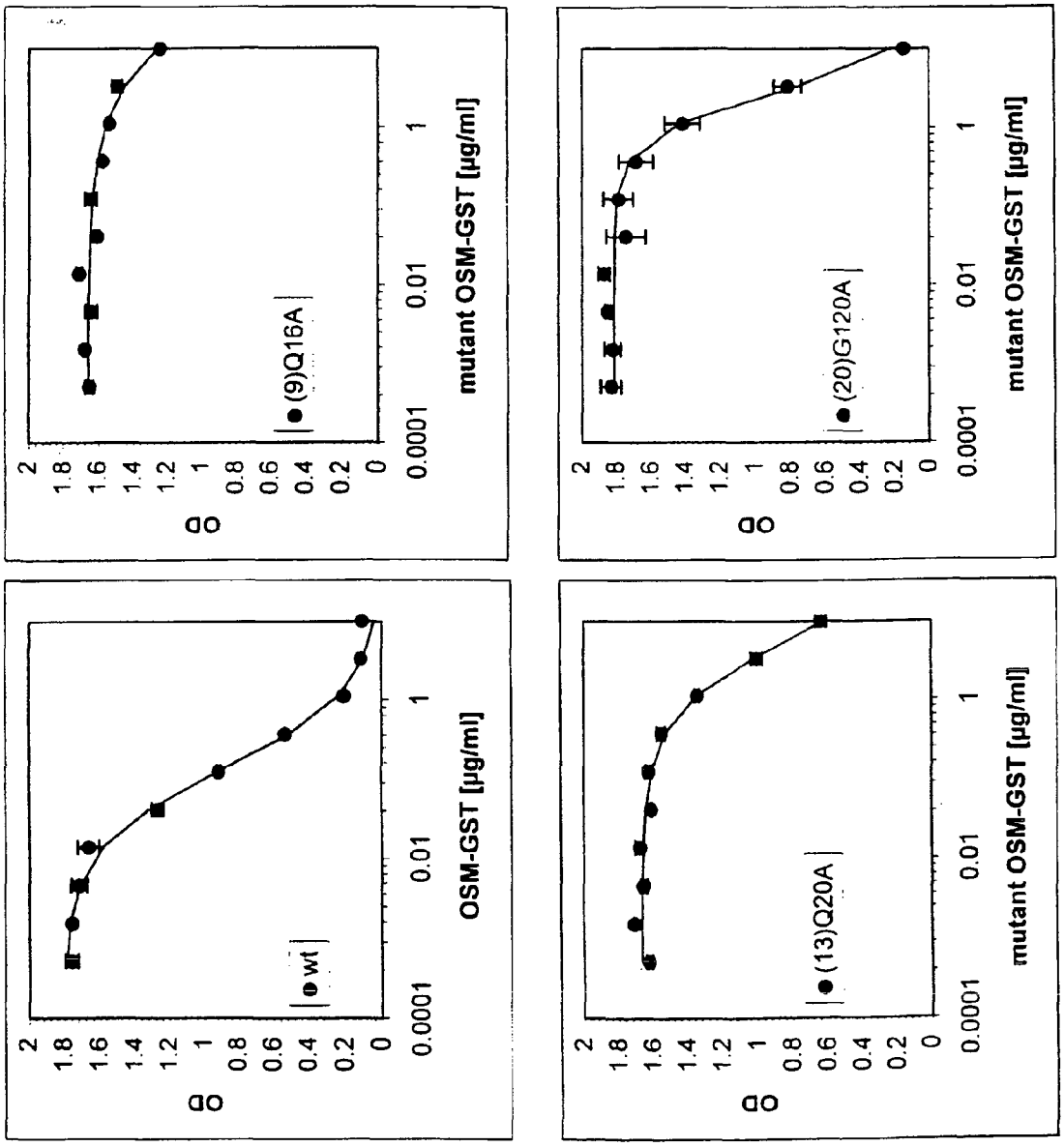
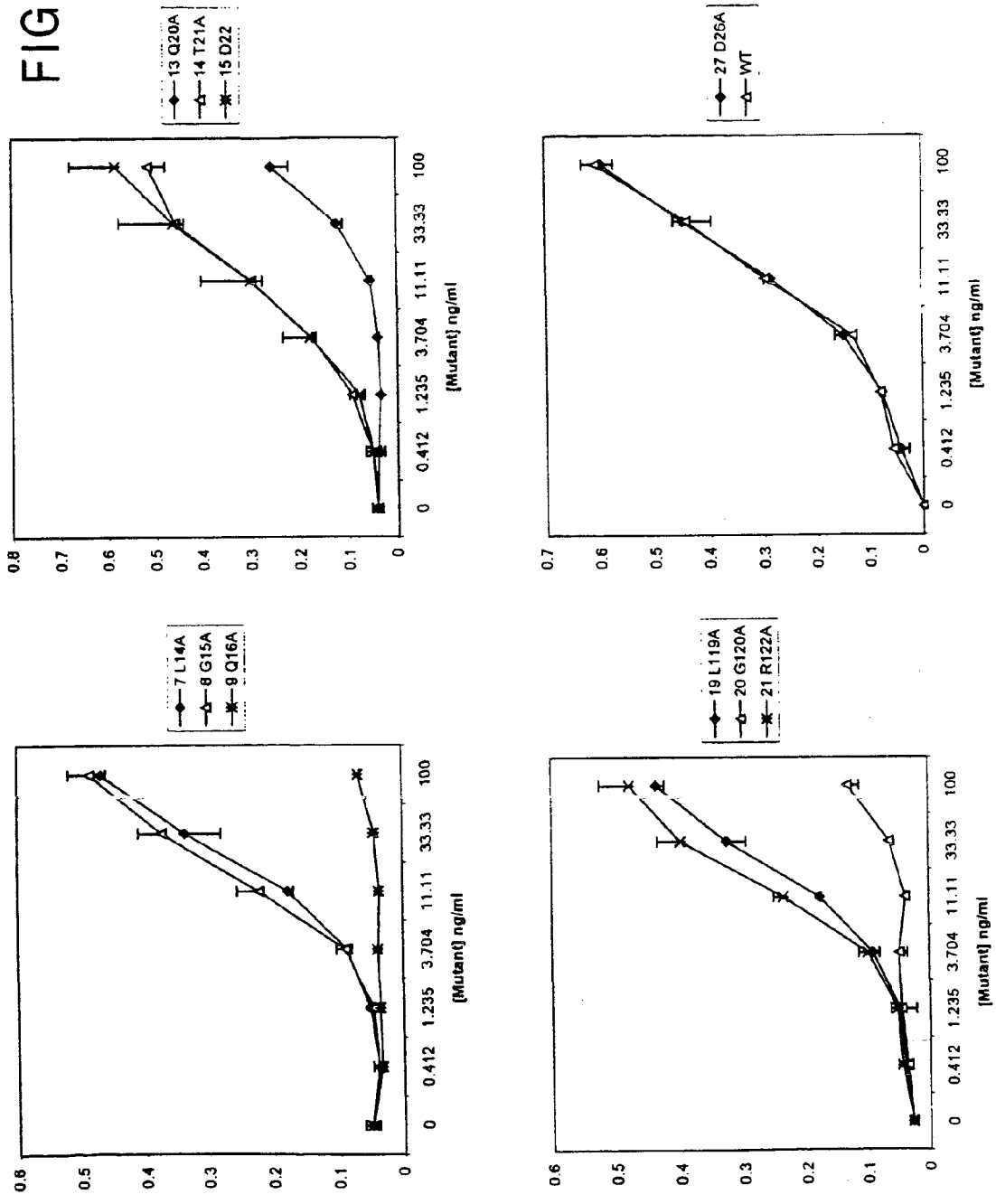

OD
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.0001
0.01
1
wt
OSM-GST [µg/ml]
(9)Q16A
mutant OSM-GST [µg/ml]
(13)Q20A
(20)G120A

FIG.13


13 Q20A
14 T21A
15 D22
27 D26A
WT
7 L14A
8 G15A
9 Q16A
19 L119A
20 G120A
21 R122A
[Mutant] ng/ml
0
0.412
1.235
3.704
11.11
33.33
100