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(72) Inventeur/Inventor:
HOUCK, JOHN C., US

(73) Propriétaire/Owner:
MOWYCAL LENDING, LLC, US

(74) Agent: DIMOCK STRATTON LLP

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(54) Title: SMALL PEPTIDES AND METHODS FOR TREATMENT OF ASTHMA AND INFLAMMATION

(57) Abrégé/Abstract:
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(71) Applicant: HISATEK, LLC [US/US]; 530 36th Avenue E., Seattle, WA 98112 (US).

(72) Inventor: HOUCK, John, C.; 3219 N.E. 68th Street, Seattle, WA 98115 (US).

(74) Agents: NEUNER, George, W. et al.; Dike, Bronstein, Roberts & Cushman LLP, 130 Water Street, Boston, MA 02109 (US).

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A pharmaceutical composition is described as an admixture of a pharmacological carrier and a peptide having the formula: f–Met–Leu–X. X is selected from the group consisting of Tyr, Tyr–Phe, Phe–Phe and Phe–Tyr. Also described are methods for inhibiting the degranulation of mast cells and for treating inflammation in a patient, for example, where the inflammation is a result of a disease selected from the group consisting of asthma, rheumatoid arthritis and anaphylaxis. In addition, methods are described for inhibiting the release of cytokines in a patient, for inhibiting the release of histamines in a patient, for inhibiting the release leukotrienes in a patient, for reducing adhesion, migration and aggregation of lymphocytes, eosinophils and neutrophils to a site of inflammation in a patient, for reducing the production of IgE antibodies at site of inflammation in a patient, and for inhibiting increased vascular permeability at site of inflammation in a patient. The methods use the described pharmaceutical composition.

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SMALL PEPTIDES AND METHODS FOR TREATMENT OF ASTHMA AND INFLAMMATION

FIELD OF THE INVENTION

This invention relates to small peptides having mast cell degranulation inhibition activity and to methods for treating inflammation, and particularly to N-formyl-methionyl peptides useful for the treatment of inflammation. More particularly, the invention relates to methods of treating diseases or conditions involving mast cell degranulation including, for example, asthma, rheumatoid arthritis and anaphylaxis.

BACKGROUND OF THE INVENTION

Asthma is a complex disorder. Both hereditary and environmental factors -- allergies, viral infections, irritants -- are involved in the onset of asthma and in its inflammatory exacerbations. More than half of asthmatics (adults and children) have allergies; indeed, allergy to house dust mite feces is a major factor in the development of the disease and in the occurrence of exacerbations. Infection with respiratory syncytial virus during infancy is also highly associated with the development of asthma, and viral respiratory infections often trigger acute episodes.

The introduction three decades ago of bronchodilating $beta_2$-agonists -- adrenergic agonists selective for the $beta_2$ receptor -- revolutionized the treatment of asthma. These agents proved to be more potent and longer acting (4-6 hours) than the nonselective adrenergic receptor agonists such as isoproterenol, which stimulate both alpha- and beta-adrenergic receptors. $Beta_2$-agonists give rapid symptomatic relief and also protect against acute bronchoconstriction caused by stimili such as exercise or the inhalation of frigid air. Frequency of use can also serve as an indicator of asthma control.

Recently, an extra long- acting beta$_2$-agonist-salmeterol (duration up to 12 hours) was introduced in the United States. Salmeterol is so potent that it may mask inflammatory signs; therefore, it should be used with an anti-inflammatory.

Theophylline is a relatively weak bronchodilator with a narrow therapeutic margin (blood level monitoring is recommended to avoid toxicity) and a propensity for drug interactions (competition for hepatic cytochrome P450 drug-metabolizing enzymes alters plasma levels of several important drugs metabolized by that same system).

Moderate asthma is treated with a daily inhaled anti-inflammatory-corticosteroid or mast cell inhibitor (cromolyn sodium or nedocromil) plus an inhaled beta$_2$-agonist as needed (3-4 times per day) to relieve breakthrough symptoms or allergen- or exercise-induced asthma. Cromolyn sodium and nedocromil block bronchospasm and inflammation, but are usually effective only for asthma that is associated with allergens or exercise and then, typically, only for juvenile asthmatics. Inhaled corticosteroids improve inflammation, airways hyperreactivity, and obstruction, and reduce the number of acute exacerbations. However, it takes a month before effects are apparent and up to a year for marked improvement to occur. The most frequent side effects are hoarseness and oral candidiasis. More serious side effects have been reported --partial adrenal suppression, growth inhibition, and reduced bone formation -- but only with the use of higher doses. Beclomethasone, triamcinolone, and flunisolide probably have a similar mg-for-mg potency; the newer approvals budesonide and fluticasone are more potent and reportedly have fewer systemic side effects.

Even patients with mild disease show airways inflammation, including infiltration of the mucosa and epithelium with activated T cells, mast cells, and eosinophils. T cells and mast cells release cytokines that promote eosinophil

growth and maturation and the production of IgE antibodies, and these, in turn, increase microvascular permeability, disrupt the epithelium, and stimulate neural reflexes and mucus-secreting glands. The result is airways hyperreactivity, bronchoconstriction, and hypersecretion, manifested by wheezing, coughing, and dyspnea.

Traditionally, asthma has been treated with oral and inhaled bronchodilators. These agents help the symptoms of asthma, but do nothing for the underlying inflammation. Recognition during the last 10 years of the importance of inflammation in the etiology of asthma has led to the increased use of corticosteroids, but many patients continue to suffer from uncontrolled asthma.

Scientists have determined that the leukotrienes (of which there are A, B, C, D, and E subtypes) plays a crucial role in asthma. They cause airways smooth muscle spasm, increased vascular permeability, edema, enhanced mucus production, reduced mucociliary transport, and leukocyte chemotaxis.

Like related prostaglandin compounds, leukotrienes are synthesized from arachidonic acid in the cell membrane. Arachidonic acid in mast cells, eosinophils, macrophages, monocytes, and basophils is formed from membrane phospholipids by the activation of phospholipase A2. After its formation, arachidonic acid undergoes metabolism via two major pathways: the cyclooxygenase pathway (which produced various prostaglandins and thromboxanes) and the 5-lipoxygenase pathway (which produces leukotrienes). A schematic of arachidonic acid metabolism is illustrated in FIG. 4. The prostaglandins, thromboxanes, and leukotrienes are known collectively as eicosanoids.

Anti-leukotrienes are members of a heterogeneous class of anti-asthma agents with the potential to interfere with the initial steps in the inflammatory

cascade. Leukotrienes are inflammatory substances related to prostaglandins; both are generated from arachidonic acid in cell membranes. After arachidonic acid in mast cells, eosinophils, macrophages, monocytes, and basophils is formed, it is metabolized via two major pathways: (1) a cycloxygenase pathway (which produces prostaglandins and thromboxanes) and (2) the 5-lipoxygenase pathway, which produces leukotrienes in the cytoplasma. The leukotrienes are well known in medical science as the slow reacting substance of anaphylaxis ("SRS-A"). Leukotrienes play an important role in bronchial inflammation. They induce migration, adhesion and aggregation of various white blood cells (e.g., neutrophils, eosinophils, and monocytes) to blood vessels, increase capillary permeability, and cause bronchial and vessel smooth muscle constriction. The results include interstitial edema, leukocyte chemotaxis, mucus production, mucociliary dysfunction, and bronchospasm in the lungs. Certain classes of leukotrienes, for example, the cysteinyl leukotrienes (LTD_4), are particularly potent bronchoconstrictors, being approximately 100 to 1,000 times more active than histamine. Leukotrienes, including cysteinyl leukotrienes, are released from mast cells during degranulation.

A number of anti-leukotrienes that either block leukotriene receptors or prevent leukotriene synthesis by blocking the enzyme 5-lipoxygenase are under investigation and in commercial use. The leukotriene inhibitors are heterogeneous in action: some block 5-lipoxygenase directly, some inhibit the protein activating 5-lipoxygenase, and some displace arachidonate from its binding site on the protein. The leukotriene antagonists, by contrast, block the receptors themselves that mediate airways hyperactivity, bronchoconstriction, and hypersecretion.

Human lung mast cells produce tumor necrosis factor (TNF), IL-4 and IL-5 after IgE stimulation *in vitro* (*Chest* 1997; 112:523-29). Immunohistochemical analysis in endobronchial biopsy specimens has confirmed this together with IL-6 production. Further, mast cell counts and

TNF are statistically more significant in asthmatics when compared to normal subjects. TNF and IL-4 can potentiate up-regulation of the expression of vascular cell adhesion molecule-1 (VCAM-1) --an adhesion molecule of the immunoglobin super family-- in the endothelial layer of the bronchial vasculature. Eosinophils, basophils and mononuclear cells display the very late activation antigen 4 (VLA-4) integrin on their cellular surfaces, which interacts with VCAM-1. Thus, through the interaction VLA-4/VCAM-1, TNF and IL-4 facilitate the recruitment of circulating leukocytes. The capacity of mast cells to release preformed cytokines (TNF) on IgE-mediated stimulus or to rapidly synthesize others (IL-4, IL-5) could be the initial event leading to bronchial inflammation. In fact, the induction and activation of TH2 clones, through a further production of cytokines, facilitates the activation and recruitment of the eosinophils, which act as the terminal effectors of the inflammatory reaction. In turn, the cytokines produced by leukocytes (TH2 cells, in particular) profoundly affect the development, activation, and priming of mucosal mast cells, thus promoting a positive proinflammatory loop. The recent findings that human mast cells produce IL-8 and that murine pulmonary-derived mast cells express both chemokines, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1. This suggests that, besides the cytokines classically involved in leukocyte recruitment (IL-4, IL-5, TNF), mast cells also elaborate additional, potent chemoattractants in the airways, acting on eosinophils and polymorphonuclear leukocytes (IL-8). Moreover, because chemokines acting as histamine-releasing factors elicit mast cell degranulation, they may further sustain an autocrine activating loop.

The mast cells also play a key role in B-cell growth to provide the cell contact (like basophils) that is required, along with IL-4, for IgE synthesis in vitro, which suggests that mast cells may directly regulate the production of IgE independently of T-cells, and may, upon IgE cross-linking, generate a sufficient amount of IL-4 to initiate a local TH2 response, the subset of T-cells considered to play a central role in atopic asthma. Moreover, mast cells can also act as an

antigen-presenting cell to T-lymphocytes, suggesting an even larger role for mast cells in the immune network of asthma.

Inhibition of mast cell degranulation by N-formyl-methionyl-leucyl- phenylalanine [SEQ ID NO: 1] was reported in Inflammation, Vol. 5, No.1, pp. 13-16(1981). There, it was reported that two structurally different chemotactic peptides, i. e., pepstatin and N-formyl-methionyl-leucyl-phenylalanine [SEQ ID NO: 1], inhibit the increase in vascular permeability produced byintradermal injection of 40/80, anti-rat IgE serum, or macromolecular anionic permeability factor isolated from calf lung in rat skin. It also has been reported that these peptides appear to act directly on the mast cells.

Because of the importance of treating inflammatory diseases in humans, particularly, for example, asthma, arthritis and anaphylaxis, new bioactive compounds having fewer side effects are continually being sought. The inhibition of mast cell degranulation by the intervention of novel peptides of the present invention within the context of the asthma inflammatory process is visually depicted in FIG. 4.

SUMMARY OF THE INVENTION

The present invention provides novel pharmaceutical compositions containing in a suitable pharmacological carrier a N-formyl-methionyl-leucyl ("Met-Leu") [SEQ ID NO: 2] peptide having mast cell degranulation inhibition activity. Particularly useful such peptides are those having the formula f-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr. Such peptides are useful for treating inflammation, and particularly in treating inflammation connected with asthma, arthritis and anaphylaxis. These peptides also are useful for treating chronic obstruction pulmonary disease and chronic inflammatory bowel disease.

In accord with the present invention, a method for treating inflammation in a mammal comprises administering to the mammal an anti-inflammatory effective amount of a peptide having the formula f-Met-Leu-X where X [SEQ ID NO: 3] is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr. For treating inflammation connected with asthma, a preferred mode of administration is by inhalation. For treating inflammation connected with arthritis, a preferred mode of administration is topical application or intradermal injection, using a suitable pharmacological carrier.

The present invention also provides a method for inhibiting the degranulation of mast cells. The method comprises contacting mast cells with a degranulation inhibiting amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

Further, the present invention also provides a method for inhibiting the release of cytokines, histamines and leukotrienes. The method for inhibiting the release of cytoldnes comprises administering to the patient a cytokine release inhibiting effective amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr. The method for inhibiting the release of histamines comprises administering to the patient a histamine release inhibiting effective amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr. The method for inhibiting the release of leukotrienes comprises administering to the patient a leukotriene release inhibiting effective amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

In accord with another embodiment, the invention provides a method for reducing adhesion, migration and aggregation of lymphocytes, eosinophils and

neutrophils to a site of inflammation in a patient. The method comprises administering to the patient a inhibiting therapeutically effective amount of a peptide having the formula f-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

Also, the invention provides a method for reducing the production of IgE antibodies and reducing or blocking IgE cross-linking at the site of inflammation in a patient. The method comprises administering to the patient an IgE antibody production inhibiting effective amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr
Phe, Phe-Phe and Phe-Tyr.

In addition, the present invention provides a method for inhibiting increased vascular permeability at site of inflammation in a patient. The method comprises administering to the patient a vascular permeability inhibiting effective amount of a peptide having the formulaf-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

In certain preferred embodiments of the present invention, patients having chronic inflammation can benefit by administering the peptide of the present invention in combination with another active ingredient. Particularly useful other active ingredients for such combination in accord with the present invention are, for example, antileukotrienes, beta2 agonists, corticosteroids, and the like.

BRIEF DESCRIPTION OF DRAWINGS

FIG.1 is a log dose response curve illustrating area of capillary permeability for various concentrations of Compound 48/80.

FIG. 2 is a dose response curve for inhibition of capillary permeability by various concentrations of f-Met-Leu-Phe [SEQ ID NO: 1].

FIG. 3 is a dose response curve for inhibition of capillary permeability by various concentrations of a preferred peptide of the present invention.

FIG. 4 is a schematic illustration of the major pathways for arachidonic acid metabolism further illustrating inhibition of mast cell degranulation.

FIGs. 5A and-5B are schematic illustrations of the different protocols of Standard(5A) and Resolution (5B) used in The OVA-induced Bronchial Asthma Mouse Model.

FIGs. 6a-6D are micrographs illustrating the comparative histopathology of a treatment with a compound of the present invention inhibiting the OVA induced asthma in treated mice and control mice.

FIG. 7is a histogram showing the results for treatment in accord with the present invention on formation of mucus plugs in a murine asthma model.

DETAILED DESCRIPTION OF THE INVENTION

In accord with the present invention, certain small peptides having the formula f-Met-Leu-X [SEQ ID NO: 3] where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr have been found to have surprising activity for inhibiting the degranulation of mast cells. As a result, such peptides inhibit the release of cytokines (such as, for example, TNF), as well as histamines and leukotrienes and they are useful for treatment of inflammation, which can result from a variety of ailments such as, for example, asthma, arthritis and anaphylaxis. Such peptides also are useful in treating chronic obstruction pulmonary disease and chronic inflammatory bowel disease.

In accord with preferred embodiments of the present invention, the peptides also can reduce the infiltration of eosinophils, basophils and

neutrophils into inflammatory tissues. Lymphocytes, eosinophils, and neutrophils do not exhibit chemotaxis in response to preferred peptides of the present invention. Further, preferred compounds of the present invention exhibit no toxicity to vital organs such as heart, liver and lungs.

The peptides of this invention can be prepared by conventional small peptide chemistry techniques. The peptides when used for administration are prepared under aseptic conditions with a pharmaceutically acceptable carrier or diluent.

Doses of the pharmaceutical compositions will vary depending upon the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000 μg/kg a day, more preferably 1 to 10,000 μg/kg. Most preferred dosages range from about 1 to 100 μg/kg, more preferably from about 1 to 10 μg/kg of body weight. Doses are typically administered from once a day to every 4-6 hours depending on the severity of the condition. For acute conditions, it is preferred to administer the peptide every 4-6 hours. For maintenance or therapeutic use, it may be preferred to administer only once or twice a day. Preferably, from about 0.18 to about 16 mg of peptide are administered per day, depending upon the route of administration and the severity of the condition. Desired time intervals for delivery of multiple doses of a particular composition can be determined by one of ordinary skill in the art employing no more than routine experimentation.

Routes of administration include oral, parenteral, rectal, intravaginal, topical, nasal, ophthalmic, direct injection, etc. In a preferred embodiment, the peptides of this invention are administered to the patient in an anti-inflammatory effective amount or in a dosage that inhibits degranulation of mast cells. An exemplary pharmaceutical composition is a therapeutically effective amount of a peptide in accord with the present invention that provides

anti-inflammatory effect or that inhibits degranulation of mast cells, typically included in a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" as used herein, and described more fully below, includes one or more compatible solid or liquid filler diluents or encapsulating substances that are suitable for administration to a human or other animal. In the present invention, the term "carrier" thus denotes an organic or inorganic ingredient, natural or synthetic, with which the molecules of the invention are combined to facilitate application. The term "therapeutically-effective amount" is that amount of the present pharmaceutical compositions, which produces a desired result or exerts a desired influence on the particular condition being treated. Various concentrations may be used in preparing compositions incorporating the same ingredient to provide for variations in the age of the patient to be treated, the severity of the condition, the duration of the treatment and the mode of administration.

The carrier must also be compatible. The term "compatible", as used herein, means that the components of the pharmaceutical compositions are capable of being commingled with a small peptides of the present invention, and with each other, in a manner such that does not substantially impair the desired pharmaceutical efficacy.

The small peptides of the invention are typically administered *per se* (neat). However, they may be administered in the form of a pharmaceutically acceptable salt. Such pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene-sulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulfonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium

or calcium salts of the carboxylic acid group. Thus, the present invention provides pharmaceutical compositions, for medical use, which comprise peptides of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients.

The compositions include those suitable for oral, rectal, intravaginal, topical, nasal, ophthalmic or parenteral administration, all of which may be used as routes of administration using the materials of the present invention. Pharmaceutical compositions containing peptides of the present invention may also contain one or more pharmaceutically acceptable carriers, which may include excipients such as stabilizers (to promote long term storage), emulsifiers, binding agents, thickening agents, salts, preservatives, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the peptide of this invention, its use in pharmaceutical preparations is contemplated herein. Supplementary active ingredients can also be incorporated into the compositions of the present invention.

Compositions suitable for oral administration are preferred for treatment of asthma. Typically, such compositions are prepared as an inhalation aerosol, nebule, syrup or tablet. Compositions suitable for topical administration are preferred for treatment of arthritis, although oral compositions also can be convenient. Typically, such topical compositions are prepared as a cream, an ointment, or a solution.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Methods typically include the step of bringing the active ingredients of the

invention into association with a carrier that constitutes one or more accessory ingredients.

Compositions of the present invention suitable for inhalation administration may be presented, for example, as aerosols or inhalation solutions. An example of a typical aerosol composition consists of the desired quantity of microcrystalline peptide suspended in a mixture of trichloromonofluoromethane and dichlorodifluoromethane plus oleic acid. An example of a typical solution consists of the desired quantity of peptide dissolved or suspended in sterile saline (optionally about 5 % v/v dimethylsulfoxide ("DMSO") for solubility), benzalkonium chloride, and sulfuric acid (to adjust pH).

Compositions of the present invention suitable for oral administration also may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the peptide of the invention, or which may be contained in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion. An example of a tablet formulation base includes corn starch, lactose and magnesium stearate as inactive ingredients. An example of a syrup formulation base includes citric acid, coloring dye, flavoring agent, hydroxypropylmethylcellulose, saccharin, sodium benzoate, sodium citrate and purified water.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the molecule of the invention, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-

butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In aqueous solutions, up to about 10 % v/v DMSO or Trappsol™ can be used to maintain solubility of some peptides. Also, sterile, fixed oils may be conventionally employed as a solvent or suspending medium. For this purpose, a number of fixed oils can be employed including synthetic mono-or diglycerides. In addition, fatty acids (such as oleic acid or neutral fatty acids) can be used in the preparation of injectibles. Further, Pluronic™ block copolymers can be formulated with lipids at 4 C for compound injection on a time release basis from solid form at 37 C over a period of weeks or months.

Compositions suitable for topical administration may be presented as a solution of the peptide in Trappsol or DMSO, or in a cream, ointment, or lotion. Typically, about 0.1 to about 2.5 % active ingredient is incorporated into the base or carrier. An example of a cream formulation base includes purified water, petrolatum, benzyl alcohol, stearyl alcohol, propylene glycol, isopropyl myristate, polyoxyl 40 stearate, carbomer 934, sodium lauryl sulfate, acetate disodium, sodium hydroxide, and optionally DMSO. An example of an ointment formulation base includes white petrolatum and optionally mineral oil, sorbitan sesquioleate, and DMSO. An example of a lotion formulation base includes carbomer 940, propylene glycol, polysorbate 40, propylene glycol stearate, cholesterol and related sterols, isopropyl myristate, sorbitanpalmitate, acetyl alcohol, triethanolamine, ascorbic acid, simethicone, and purified water.

<u>The Rat Skin Model for Determination of Inhibition of Mast Cell Degranulation</u>

Allergy induced asthma results from exposure-of substances (allergens) to which an organism has become hypersensitized. Exposure to allergen results in degranulation of mast cells in the lung, releasing leukotrienes and histamines. In response to the release of leukotrienes and histamines, capillary permeability is dramatically increased and blood plasma leaks from the capillaries into the surrounding tissues. Respiratory symptoms resulting from

such an exposure range from mild (itching and sneezing) to potentially fatal (asthma), including in extreme chronic cases death by anaphylaxis.

To demonstrate this phenomenon experimentally, rat skin is substituted for lung. In this model, the blood plasma of the experimental rat is labeled with the dye trypan blue. This soluble dye is carried in the bloodstream as a passive marker of plasma itself, and is excluded from live cells. Intact blood vessels, including the capillary system, retain this dye under normal circumstances. A compound, which induces degranulation of mast cells (resulting in leukotriene and histamine release), is injected into the skin to simulate allergen-induced degranulation. In these experiments, Compound 48/80 was used for this purpose. In the events following leukotriene and histamine release, capillary permeability is increased, and plasma, dyed blue, leaks from capillaries and dyes the skin surrounding the injection site blue. The area of bluing is a measure of the amount of Compound 48/80 injected.

A compound can be tested for "anti-leukotriene" and/or "anti-histamine" activity by mixing it with Compound 48/80 prior to injection. If the test compound inhibits leukotriene or histamine release, abn area of bluing of smaller diarneter is observed when compared to an injection sit eon the same rat into which Compound 48/50 has been injected without any of the test compound. In the case of high anti-leukotriene and anti-histamine activity, the bluing may actually be totally inhibited.

Experimental

The rat skin model was undertaken and valicated. Various peptides were tested as t a predetermined does for anti-leukotriene and/or anti-histamine activity. The does selected allowed a general comparison to f-Met-Leu-Phe [SEQ ID NO: 1], which was standard compound for comparison.

A "dose response" titration was performed for some compounds and compared with the standard compound. Observing serial decreases in the size areas of capillary permeability using serially smaller doses of the putative inhibitory compound validates the inhibition of leukotriene and/or histamine release observed in the initial predetermined dose test.

Materials and methods

Reagents were obtained from Sigma or Aldrich, with the exception of ketamine, a veterinary anaesthetic that was obtained from various veterinary suppliers. The rats used were male Sprague-Dawley breed, 220-240g at time of purchase from B&K International.

For the rat skin reaction, rats were anaesthetized with 0.25 ml 10 mg/ml ketamine. 1.0 ml trypan blue in saline (sterile filtered) was administered in a tail vein, and the back of the rat was shaved. Four intradermal injection sites per rat were used for test and control injections.

Compound 48/80 was prepared as a 1.5 mg/ml stock solution in saline. This material was found to be potentially unstable in aqueous solution and was prepared freshly each day. Serial dilutions in saline to working levels were prepared just prior to injection of each rat.

Peptides were prepared as a 23 mM stock solution in DMSO, and stored at -20°C between experiments. At the time of use, the frozen stock solutions were thawed, and appropriate aliquots added to dilutions of Compound 48/80, along with appropriate amounts of DMSO, to result in the ratio of 5 µl DMSO to 0.1 ml aqueous Compound 48/80. This resulted in a 5% solution of DMSO, necessary to maintain solubility of certain peptides. The effect of 5% DMSO was demonstrated by control experiments to be nil.

For injections,0. 1 ml Compound 40/80, +/-test compounds were injected intradermally into anaesthetized, dyed, and shaved rats. Following a 15 minute incubation, the rats were sacrificed by cervical dislocation and the back skin was evulsed and placed on a light box. An image of the backlit skin was digitized using a CCD video capture camera and compatible hardware/software. The digitized image was analyzed using a scientific graphics analysis software package, and the areas of capillary permeability (bluing) were integrated and digital values were obtained for further analysis.

A dose response curve was generated using Compound 48/80 at various doses from ca. 0.0 1ig0.0 1ig through ca. 15g. The results are shown in FIG. 1. Wide variability was noted in the diameter of areas of capillary permeability for a given dose of Compound 48/80 based upon rat-to-rat variations (e. g., thickness of skin). A dose of 0.15ug of Compound 48/80 was selected for conducting further tests.

Example 1

A dose response curve was prepared for the standard compound, f-Met-Leu-Phe, using the selected dose of 0.15g0.15g Compound 48/80. Doses of 0 to about 230 nMof f-Met-Leu-Phe [SEQ ID NO: 3] were tested and the results are shown in FIG. 2. Inhibition of degranulation induced by Compound 48/80 was clearly shown.

Examples 2-11

Several f-Met-Leu [SEQ ID NO: 2] peptides were tested for inhibition of induced degranulation in the rat skin model using 100 nanomoles of the test peptide and a dose of 0.15us Compound 48/80. An intrinsic zero-peptide-dose 48/80 control was included in each rat for each experiment, and the % of inhibition was expressed in relative terms to this control (0 % inhibition). The percent mast cell degranulation produced by 48/80 was also determined. The results are tabulated below.

Table 1

Example	Peptide	% Inhibition	%Degranulation*	[SEQ ID NO:]
2	f-Met-Leu-Phe (prior art)	30	60	1
3	N-acetyl-Met-Leu-Phe	0	98	4
4	N-t-BOC-Met-Leu-Phe	0-	-	5
5	f-Met-Leu- (iodo) Phe	0	-	6
6	f-Met-Leu-Phe (benzylamide)	0	-	7
7	f-Met-Leu-Phe-Lys	0	-	8
8	f-Met-Leu-Phe (methyl ester)	0-	-	9
9	f-Met-Leu-Phe-Phe	100	1-3	10
10	f-Met-Leu-Tyr	55	30	11
11	f-Met-Leu-Tyr-Tyr	0	-	12

Example 12

A dose response curve was prepared for f-Met-Leu-Phe-Phe [SEQ ID NO: 10] using the selected dose of 0.15g Compound 48/80. Doses of 0 to about 230 nM of f-Met-Leu-Phe-Phe [SEQ ID NO: 10] were tested and the results are shown in FIG. 3. Surprisingly remarkable inhibition of degranulation induced by Compound 48/80 was clearly shown. The inhibition of induced degranulation for f-Met-Leu-Phe-Phe [SEQ ID NO: 10] was unexpectedly substantially better than that of the standard compound f-Met-Leu-Phe.[SEQ ID NO: 10].

The OVA-induced Bronchial Asthma Mouse Model
for Inhibition of Mast Cell Degranulation

Asthma is a complex disease, which is characterized by spontaneous exacerbation of airways obstruction and persistent bronchial hyperresponsiveness. Chronic infiltration with activated T-lymphocytes, eosinophils and macrophages/monocytes of the airway submucosa is another established feature. Inflammatory mechanisms, with expression of cytokines,

and the release of inflammatory mediators, underlie the pathogenesis of bronchoconstriction and bronchial hyperresponsiveness. However, much of the pathogenic mechanism remains unclear, e. g., the mechanisms that induce persistence of symptoms and chronic inflammation and the interventions necessary to control and prevent the disease.

It has long been recognized that a single inhaled allergen challenge can induce an acute increase in airway responsiveness in some individuals and animal models. However, repeated allergen inhalations have demonstrated more pronounced, consistent, and prolonged increases in airway responsiveness. This mouse model of long-term repeated inhalations of allergen has been used to study the long term effect of allergic diseases in the lung, and to delineate the cells, mechanisms, molecules, and mediators involved in the induction of airway hyperresponsiveness of lung in humans.

Materials and Methods

Reagents: Crystalline OVA was obtained from Pierce Chem. Co. (Rockford, IL) aluminum potassium sulfate (alum) from Sigma Chem. Co. (St. Louis, MO), pyrogen-free distilled water from Baxter, Healthcare Corporation (Deerfield, IL), 0.9% sodium chloride (normal saline) from Lymphomed(Deerfield, IL) and TrappsolHPB-L100 (aqueoushydroxypropyl beta cyclodextrin; 45 wt/vol % aqueous solution) from Cyclodextrin TechnologiesDevelopment, Inc. (Gainesville, FLA). The OVA (500μg/ml in normal saline) was mixed with equal volumes of 10% (wt/vol) alum in distilled water. The mixture (pH 6.5 using 10 NNaOH) after incubation for 60 minutes at room temperature underwent centrifugation at 750 g for 5 minutes; the pellet was resuspended to the original volume in distilled water and used within one hour.

The selective 5-lipoxtgenase inhibitor, Zileuton™ (N- [l-benzo [b] thien-ylethyl]-N-hydroxyurea; J. Pharmacol Exp Ther. 1991; 256: 929-937), was kindly provided by Drs. Bell and George W. Carter (Abbott Laboratories, Abbott

Part, IL). Zileuton™ was dissolved in Trappsol. TM. Histatek, Inc. (Seattle, WA) provided the mast cell degranulation inhibitor, f-Met-Leu-Phe-Phe [SEQ ID NO: 10] ("HK-X").

Female BALB/c Once (6-8 wk of age at purchase; D and K, Seattle WA) were housed under conventional conditions for the studies.

Allergen Immunization/Challenge Protocols: Mice received an i.p. injection of 0.2 ml(100µg) of OVA with alum on the different protocols of Standard (FIG. 5A) and Resolution (FIG. 5B) (*J.Exp Med.* 1996; 184: 1483-1494). According to the different protocols, mice were anesthetized with 0.2 ml i.p. of ketamine (0.44 mg/ml)/xylazine (6.3 mg/ml) in normal saline before receiving an intranasal (i. n.) does of 100µgOVA in 0.05 ml normal saline and an i. n. dose of 50 g OVA in 0.05 ml normal saline separately on different days. Two control groups were used. Accordingly, the first group received normal saline with alum i. p. and normal saline without alumin.; the second group received OVA with alum i. p., OVA without alum i. n., and normal saline, alone.

Histology

The trachea and left lung (the right lung is used for bronchoalveolar lavage ("BAL")) were obtained and fixed in 10% neutral formaldehyde solution at room temperature for 6-15h. After being embedded in paraffin, the tissues were cut into 5-um sections and processed with the different staining or immunolabling further. Discombe's eosinophil staining was used for counting the cell numbers with the counterstain of methylene blue. The eosinophil number per unit airway area (2,200 pm2) was determined by morphometry (*J. Pathol.* 1992; 166: 395-404; *Am Rev Respir Dis.* 1993; 147: 448-456). Fibrosis was identified with the Masson's trichrome staining. Airway mucus was identified by the following staining method: methylene blue, hematoxylin and eosin, mucicarmine, alcian blue, and alcian blue/periodic acid-Schiff (PAS) reaction (Troyer, H., "*Carbohydrates"inPrinciples and Techniques of*

Histochemistry, Little, Brown and Company, Boston, MA, 1980: 89-121; Sheehan, D.C., et al., "Carbohydrates" in *Theory and Practice of Histotechnology*, Battle Press, Columbus, OH, 1980: 159-179). Mucin was stained with mucicarmine solution; metanil yellow counterstain was employed. Acidic mucin and sulfated mucosubstances were stained with alcian blue, pH 2.5; nuclear fast red counterstain was used. Neutral and acidic mucosubstances were identified by alcian blue, pH 2.5, and PAS reaction. The degree of mucus plugging of the airways (0.5-0.8 mm in diameter) was also assessed by morphometry. The percent occlusion of airway diameter by mucus was classified on a semiquantitative scale from 0 to 4+ as described in Figure Legends. The histologic and morphometric analyses were performed by individuals blinded to the protocol design.

Pulmonary Function Testing

On day 28, 24 hours after the last i.n. administration of either normal saline or OVA, pulmonary mechanics to intravenous infusion of methacholine were determined in mice in vivo by a plethysmographic method, which was modified from that previously described (10, 1958; 192: 364-368; *J. Appl. Physiol.* 1988; 64: 2318-2323; *J. Exp. Med.* 1996; 184: 1483-1494). At the completion of pulmonary function testing, each mouse was exsanguinaetd by cardiac puncture and the lung tissue with trachea was obtained for the further analysis.

Bronchoalveolar Lavage

After tying off the left lung at the mainstem bronchus, the right lung was lavaged three times with 0.4 ml of normal saline. Bronchoalveolar lavage (BAL) fluid cells from a 0.05-ml aliquot of the pooled sample were counted sing a hemocytometer and the remaining fluid centrifuged at 4°C for 10 minutes at 200 g. The supernatant was stored at -70°C until eicosanoid analysis was performed. After resuspension of the cell pellet in normal saline containing 10% bovine serum albumin ("BSA"), BAL cell smears were made on glass slides.

To stain eosinophils, dried slides were stained with Discombe's diluting fluid (0.05% aqueous eosin and 5% acetone (vol/vol) in distilled water; *J.Exp. Med.* 1970; 131: 1271- 1287) for 5-8 minutes, rinsed with water for 0.5 minutes, and counterstained with 0.07% methylene blue for 2 minutes.

Assay of Airway Mucus glycoproteins

Mucus glycoproteins in BAL fluid were assayed by slot blotting and PAS staining (*Anal.Biochem*. 1989; 182: 160-164; *Am. J. Respir. Cell Mol. Biol.* 1995; 12: 296-306). Nitrocellulose membranes (0.2-pm pore size; Schleicher & Schuell, Keene, NH) were wetted in distilled water and then in normal saline before placement in a Minifold™ II 72-well slot blot apparatus (Schleicher & Schuell). The BAL fluid samples (0.05 ml) and aliquots (0.05-0.75 1) of a stock solution(2 um/ml) of human respiratory mucin glycoprotein (*Am. J.Respir. Cell Mol. Biol.* 1991; 5: 71-79) were blotted onto the nitro-cellulose membranes by water suction vacuum, and mucus glycoproteins were visualized by PAS reaction. Reflectance densitometry was performed to quantitate the PAS staining. The images were than analyzed by an image processing system described below. The integrated intensity of the PAS reactivity of the BAL samples was quantitated by comparison to the standard curve for human respiratory mucin.

Immunocytochemistry

Monoclonal antibody: CDl lc (DAB method) andMac 1 (Beringer Mannheim, ABC method with Hitomouse™ Kit, Zymed) were used to identified the inflammatory cell types, e. g., dendric cells, macrophages and lymphocytes, in/around the areas of vasculatures, airways and fibrosis.

Morphometry and Image Analysis

All the images were captured and digitized by a ScanJet™ IICX Scanner with HP DeskScan™ II software (Microsoft(g) Windows Version) (Hewlett Packard, Palo Alto, CA). This system was linked to Dell Dimension XPS™ P90

computer (Dell Corporation, Austin, TX) employing Image-Pro Plus™, version 1.1 for Windows™ software (Media Cybernetics, Silver Spring, MD). The images were assessed on a 256 gray level scale using a Dell Ultrascan™ 17ES monitor with extra high-resolution graphics mode (1.280 X 1,024 pixels, 78.9-kHz horizontal scanning frequency, 74-Hz vertical scanning frequency).

Leukotriene Inhibitor Studies

To assess the role of 5-lipoxygenase products in airway inflammation, the 5-lipoxygenase inhibitor, Zileuton™, (35 mg/kg) was given i. p. 30 minutes before each i. n. challenge on the days according to FIG. 5. In one set of animals,
Zileuton™ was also given before i. p. OVA. Zileuton™ at 35 mg/kg inhibits cysteinyl leukotriene release by-95% in passively sensitized rats given BSA antigen i.p. (*J. Pharmacol. Exp. Ther.* 1991; 256: 929-937).

Compound HK-X of the Invention

Compound HK-X was administered at 5 mg/kg and 10 mg/kg using the same procedure as described above.

Statistical Analyses

The pulmonary function data were evaluated by analysis of variance (ANOVA) using the protected least significant difference method (Statview™ II, Abacus Concepts, Berkeley, CA). This method uses a multiple t statistic to evaluate all possible pairwise comparisons and is applicable for both equal and unequal pair sizes. The other data are reported as the mean t SE of the combined experiments. Differences were analyzed for significance ($P < 0.05$) by Student's two-tailed t test for independent means.

1. Eosinophils (Tables 2A-2B)

The eosinophil numbers of the airway in OVA-treated mouse of 1-, 2- and 3-month group were significantly reduced from 44.83% to 37.40% and 19.15%, respectively ($P < 0.025$). Even though the eosinophil count is much higher in

the OVA treated group than the other two groups at the same time course ($P < 0.025$), Zileuton™ could reduced eosinophils generally through 1-3 month. However, the HK-X compound of the present invention reduced eosinophils comparably at one month, but much more beneficially at two and three months.

Table 2A: Airway Influx of eosinophils

(%)	Saline	OVA	Zileuton™	HK	P value
3 month	1.00	19. 5	10.73	-	< 0.025
2 month	1.00	37.40	11.66	-	< 0.01
1 month	1.00	44.83	15.50	14.20	< 0.001
P value	> 0.05	< 0.025	< 0.025	< 0.025	

"

-

Table 2B: Percentage of eosinophils in airway tissue

Time of Treatment	Saline	OVA	Zileuton™	HK-X
28 days	1. 0	44.8	15.8	14. 2

2. Other inflammation cells

Other inflammation cells indicates a non-specific inflammatory response following the introduction into the airway of a foreign protein. Lymphocytes were recruited into the airways, but were virtually absent in control groups. Neutrophils were recruited following OVA challenge in boot sham-sensitized and OVA-sensitized mice, although greater numbers were presented in the airways

of the OVA sensitized group. Peculiar multinucleate giant cells (fused macrophages) having crescents of nuclei around the periphery of their extensive cytoplasm, were occasionally seen. Both Langhans giant cells and globule leukocytes were observed only in animals sensitized and challenged with OVA. They were usually present in the connective tissue associated with larger airways. Plasma cells were occasionally seen in the proximity of the airways and in local lymphoid tissue.

3. Airway plug (Table 3)

Mucin: There was no difference among the three groups with the same treatment but difference time course ($P > 0.05$). The OVA-treated group had a higher score than that of the groups treated with saline, Zileuton™ ($P < 0.05$) and HK-X compound.

Table 3: Mucus plug score in airways

Time of treatment	Saline	OVA	Zileuton	HK-X
28 days	0.7	2.8	1.3	1.4
% of plug of airway	> 5%	55%	16%	19%

Asthma is a chronic inflammatory condition of the airways. In humans, once it is established, the airway hyperresponsiveness can remain stable for years. It persists apparently in the absence of allergen inhalation, detectable airway inflammation or epithelian desquamation. Thus, it may become permanent due to irreversible (or at least slowly reversible) alterations in airway

In mild asthmatics, these episodes or "attacks" are relatively infrequent and well-treated (reversed) with haled bronchodilators. Its intensity of an underlying, distinctive and chronic airway inflammation is associated, and seemingly linked, to more frequent, intense and prolonged attacks that are less reversible by bronchodilators. The reasons for this have become increasingly clear in recent years. The inflammation, which consists principally of an activated or primed infiltrate of Th2-lymphocytes, eosinophils, mast cells, and possibly platelets, causes an expansion of the perivascular ((interstitial) spaces and release of mediators/growth factors, which cause thickening of the basement membrane, epithelial damage and shedding, production of viscous mucus, and hyperplasia, priming as well as partial constriction of airway smooth muscle. All of these outcomes support an increase in airway responsiveness, which lowers the threshold for response to environmental stimuli, thus making attacks more frequent and robust.

All the above morphological changes will directly and strongly affect the pulmonary functions. In experiments on acute asthmatic mouse model and on long-term asthmatic mouse model, the significant pathophysiological changes of pulmonary functions have been observed to support the above morphological changes. Allergen inhalation was found to increase eosinophils and mast cells expression on airway and alveolar endothelium and epithelium, as well as inducing E-selection expression only on airway endothelium, and both the eosinophil infiltration and increase in airway responsiveness, and the other types of inflammatory cells (globule leukocytes and multinucleate giant cells (fused macrophages) of the Langhans type), which indicated non-specific inflammatory reaction within the asthmatic lungs.

Compound HK-X inhibits mucus accumulation in the airway of OVA-treated (OVA) and control mice. The distribution of mucus occlusion of airways was determined from sham-sensitized and saline-challenges mice (saline, n=4), and OVA-sensitized/challenged mice in the absence (OVA, n=4) or presence

(HK-X/OVA, n=8) of HK-X treatment. Mucus occlusion of airway diameter was assayed morphometrically as following: 0, no mucus; +, ~10% occlusion; ++, 30% occlusion; +++, ~60% occlusion; ++++, ~80% occlusion. 10 airways randomly distributed throughout the lungs of each mouse were assessed for mucus occlusion morphometrically.

FIGs. 6A-6D provide visual histologic evidence of the ability to of Compound HK-X to inhibit degranulation of mast cells in asthma induced rats using OVA and thereby the effect of treating asthma with Compound HK-X. FIG. 6A shows an abundance of secreted mucus in the lumen of the airway (AW) of OVA sensitized/challenged mice. FIG. 6B shows massive infiltration of the interstitial tissue by eosinophils and other inflammatory cells (noted by arrows). FIG. 6C shows that airway mucus release in the airway (AW) lumen is markedly reduced when Compound HK-X inhibitor is given before i.n. OVA. The infiltration of the interstitial tissue by eosinophils is also reduced after Compound HK-X treatment compared to OVA-challenge alone (compare FIG. 6C with FIGs. 6A and 6B). FIG. 6D shows that the airway (AW) is clear of mucus and cells in Saline-treated control mice. The bronchial epithelium is infiltrated with connective tissue cells but no leukocytes are present in the peribronchial interstitial space.

Airway macrophages showed signs of gross activation that resembled those reported in macrophages recovered in BAL fluid from allergen-challenged lungs of asthmatics (*Am. Rev. Respir. Dis.* 1987; 135: 433-440). Macrophages and dendritic cells function as antigen-presenting cells in lung and may lead, directly or indirectly, to the secretion of cytokines able to initiate phenotypic changes in airway epithelium and its peripheral sites. The stimulation of the chronic inflammation of the airway may directly induce the proliferation of airway epithelium and fibroblasts, and the consequent collagen deposit around these areas. Activated macrophages and dendric cells remained high in the

area in comparison with the other inflammatory cells during the late-stage challenges.

The airway epithelium was thickened, due largely to a marked goblet cell hyperplasia, particularly in the larger airways, but also in small and even terminal bronchioles. The ratio of goblet cells to normal, columnar, ciliated cells was greatly increased compared with control groups. Whereas control airways (both small and large) had only the occasional goblet cells, section from OVA-challenged lungs showed that 100% of large airways and part of small airways contained goblet cells as up to 88% of the total airway epithelial cells. In lungs that had not been lavaged, mucus could be seen within the goblet cell and in some airways, occasionally completely occluding the lumen. Cellular debris was enmeshed in these mucus plugs. Goblet cell hyperplasia was not seen in control groups and, therefore, could not have been due to a "non-allergic" effect of OVA, or to the intratracheal dosing technique. Some of the goblet cells in the small airways are free of this feature, indicating perhaps that the distribution of OVA within the respiratory tree had not been uniform.

FIG. 7 is a histogram of the results for treatment with Compound HK-X at doses of 5 μg/kg and 10 μg/kg on formation of mucus plugs in this murine asthma model. Both doses significantly reduce the mucus production in small airways.

Induced Type II Collagen Arthritis Mouse Model

A mouse model is used to evaluate the effect of the compounds of the present invention on the histological, radiographic and clinical appearance of induced type II collagen arthritis.

Autoimmune diseases cause significant and chronic morbidity and disability. Arthritis in its many forms is representative of a family of

autoimmune diseases. In the clinical realm, rheumatoid arthritis (RA) is the most common form of the severe arthrodysplastic disease. All clinicians agree that RA is a progressive disease.

The histopathology of arthritic lesions occurring in murine CIA share enormous similarities to that of RA in human patients. Thus, murine CIA is an accepted model to study potential therapeutic treatments of RA.

Materials and Methods

Mice: DBA/1(2) male mice weighing 25gms (Jackson Laboratories, Bar Harbor, ME or B&K Universal, Kent WA) are used for this work. This strain of mouse is susceptible to CIA by the injection of heterologous type II collagen. Bovine Collagen (BC), Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (ICFA) can be obtained from Sigma Chemical. Antigen for immunization is processed in 0.1 M acetic acid and formulated with CFA or ICFA.

Induction of Arthritis

Immunization protocol: Mice are injected with 100μg of type II collagen in CFA at predetermined intervals during the study period.

The mice are examined at predetermined intervals for the development of arthritis. Presumptive evidence of arthritis includes swelling and erythema of at least one toe joint on the front and/or rear feet on two consecutive observations.

Confirmatory diagnosis of arthritis

Histological examination of joints: The toe joints of animals sacrificed at appropriate intervals are removed, fixed, decalcified, embedded, in paraffin, sectioned, and stained for observation of general cellular and structural features and to detect cartilaginous matrix of the pannus of each joint, as

appropriate. The degree of cellularity and areas of inflammation are quantified by using digitization of histological photomicrographs and applying standard area and point counting techniques as described above.

Radiographic evaluation of toe joints is performed to detect the incidence of joint changes after immunization with type II co0llagen. A mammography imaging system has been modified for this work. The average area of soft tissue (pannus) of the joint is determined by analysis of computer digitized radiographs, along with changes in density of the adjacent hard tissues by comparison with internal standards included with each radiograph. To serve as a baseline control for the changing density of the hard tissues and areas of panni, additional mice are used over the same period and the density and area data compared. The significance of the differences in density and area for control and experimental mice is assessed using paired T-tests at each time point.

Arthritis Evaluation

Animals are observed daily for the onset of arthritis. An arthritis index is derived by grading the severity of involvement of each paw on a scale from 0 to 4. Scoring is based upon the degree of peri-articular erythema and edema, as well as deformity of the joints. Swelling of hind paws is also quantitated by measuring the thickness of the ankle from the medial to the lateral malledus with a constant tension caliper.

Experimental Design

To assess the anti-arthritic effect of Compound HK-X, the routes of administration are selected based on experience with human patients regarding the most appropriate delivery mechanism(s).

Doses of HK-X and Prednisolone: Dosages representing divergent and putatively therapeutic levels of peptide are placed in localized sites, both by

transcutaneous (TC) (absorptive) route and by injection into the foot. Direct injection into the intraarticular space is too traumatic likely to produce artifacts. Thus, injection of drug into the footpad (FP) adjacent to the intraarticular space is the chosen methodology. Control mice are also injected with Prednisolone (a potent anti-inflammatory documented in the treatment of experimental and clinical autoimmune diseases) as a positive control.

First, each mouse in a group of ten (plus controls) is injected with collagen daily for 50 days. On days 3 and 18, the mouse is injected with 5 or 10 μg/kg of Compound HK-X in a solution of 0.1 M acetic acid at 1mg/ml. On day 50, the mouse is exsanguated for histologic studies.

Then, eight groups (A-I) of ten mice each are treated according to the following specific protocol.

Group A is immunized with 1° CFA plus BC, 2° ICFA plus BC and no treatment is given (control).

Group B is immunized with 1° CFA plus BC, 2° ICFA plus BC and prednisolone is administered at 5 mg/kg starting on the day after 2° ICFA plus BC and continued for 20 days.

Group C is immunized with 1° CFA plus BC, 2° ICFA plus BC and Compound HK-X is administered TC at 4 mg/kg (high dose) starting on the day after 2° ICFA plus BC and continued for 20 days.

Group D is immunized with 1° CFA plus BC, 2° ICFA plus BC and Compound HK-X is administered TC at 0.4 mg/kg (low dose) starting on the day after 2° ICFA plus BC and continued for 20 days.

Group E is immunized with 1° CFA plus BC, 2° ICFA plus BC and Compound HK-X is administered TC at 4 mg/kg (high dose) starting on the day after 2° ICFA plus BC and continued for 20 days.

Group F is immunized with 1° CFA plus BC, 2° ICFA plus BC and Compound HK-X is administered TC at 0.4 mg/kg (low dose) starting on the day after 2° ICFA plus BC and continued for 20 days.

Group G is immunized with 1° CFA, 2° ICFA and 10 ml DMSO is administered TC starting on the day after 2° ICFA plus BC and continued for 20 days (control).

Group H is immunized with 1° CFA, 2° ICFA and 10 ml DMSO is administered FP starting on the day after 2° ICFA plus BC and continued for 20 days (control).

Group I is immunized with 1° CFA, 2° ICFA and 10 ml saline is administered FP starting on the day after 2° ICFA plus BC and continued for 20 days (control).

Animals from each group are x-rayed immediately after 2° immunization and immediately prior to sacrifice. Following sacrifice, feet are removed as appropriate and processed for histological examination. The treatment with Compound HK-X is found to reduce the degree of arthritis.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that, upon consideration of the present specification and drawings, those skilled in the art may make modifications and improvements within the spirit and scope of this invention as defined by the claims.

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<223> M at Position 1 is N-formyl-methionyl

<400> 11

Met Leu Tyr
1

<210> 12
<211> 4
<212> PRT
<213> Artificial

<220>
<223> Synthetic peptide

<220>
<221> peptide
<222> (1)..(1)
<223> M at Position 1 is N-formyl-methionyl

<400> 12

Met Leu Tyr Tyr
1

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a pharmacological carrier and a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, the peptide being present in an effective amount for at least one of inhibiting the degranulation of mast cells and for treating inflammation in a patient.

2. The pharmaceutical composition of claim 1, wherein said peptide is f-Met-Leu-Phe-Phe.

3. The pharmaceutical composition of claim 1, wherein said peptide is f-Met-Leu-Tyr.

4. The pharmaceutical composition of claim 1, wherein said carrier is selected for administration of the peptide orally.

5. The pharmaceutical composition of claim 1, wherein said carrier is selected for administration of the peptide by inhalation.

6. The pharmaceutical composition of claim 1, wherein said composition is an aerosol composition.

7. The pharmaceutical composition of claim 1, wherein said carrier is selected for administration of the peptide topically.

8. The pharmaceutical composition of claim 1, wherein said carrier is selected for administration of the peptide by tablet.

9. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing the degranulation of mast cells.

10. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form.

11. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing inflammation.

12. The use of claim 11, wherein the inflammation is a result of a disease selected from the group consisting of asthma, rheumatoid arthritis and anaphylaxis.

13. The use of claim 11, wherein the inflammation is a result of rheumatoid arthritis.

14. The use of claim 11, wherein the inflammation is a result of anaphylaxis.

15. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing cytokine release.

16. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing histamine release.

17. The use of a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing leukotriene release.

18. The use of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing adhesion, migration and aggregation of lymphocytes, eosinophils and neutrophils to a site of inflammation in a patient.

19. The use of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for inhibiting IgE cross-linking at a site of inflammation in a patient.

20. The use of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr, in ready-to-use drug form for treating or preventing increased vascular permeability at site of inflammation in a patient.

21. The use of (i) a peptide having the formula f-Met-Leu-X, wherein X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr and (ii) an additional means for the treatment of inflammation, in ready-to-use drug form for treating or preventing increased vascular permeability at site of inflammation in a patient.

22. The use of claim 21, wherein the additional active ingredient is selected from the group consisting of anti-leukotrienes, beta2 agonists and corticosteroids.

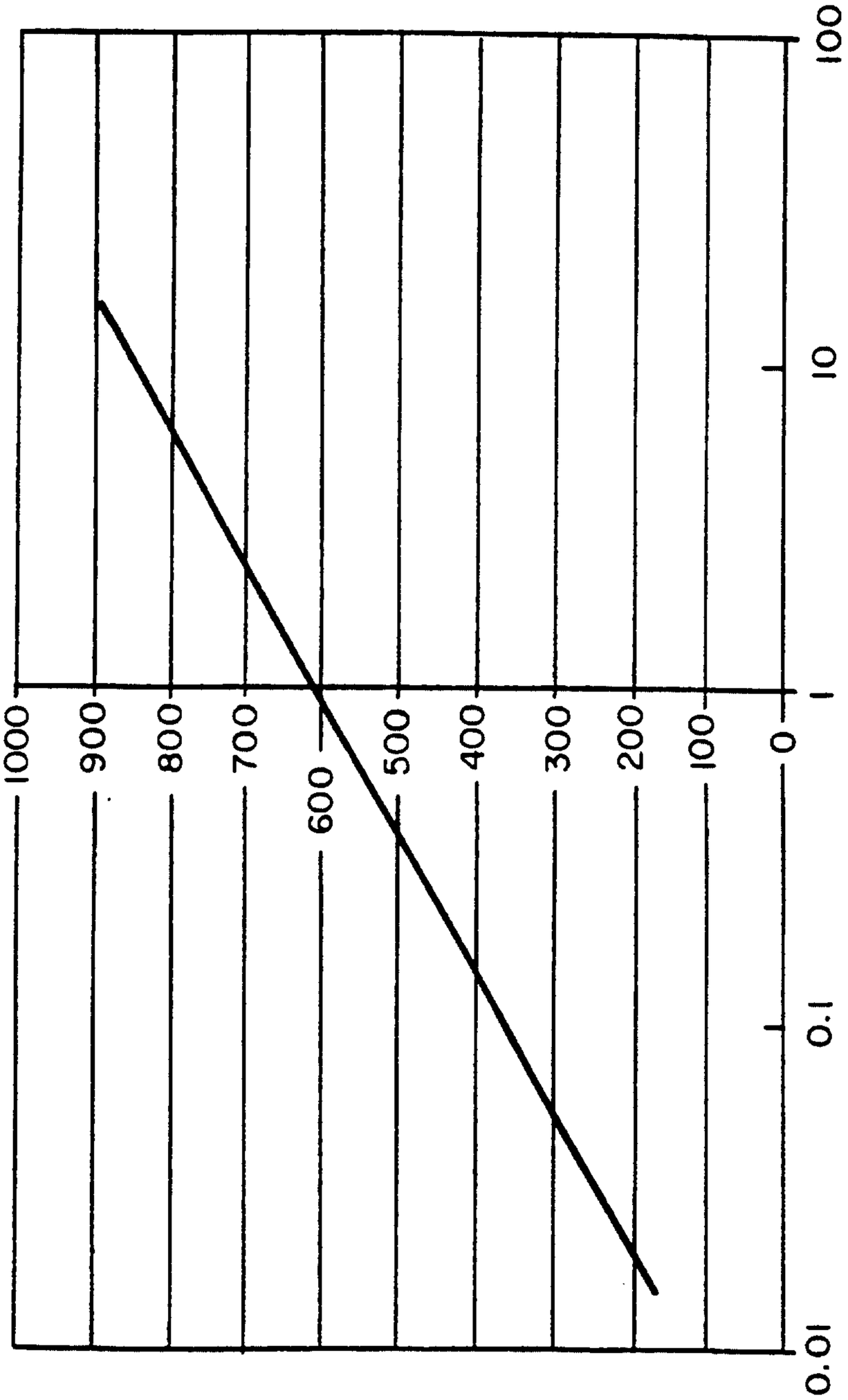
100
10
1
0.1
0.01
0
100
200
300
400
500
600
700
800
900
1000
log 48/80 concentration
area of capillary permeability

FIG. 1

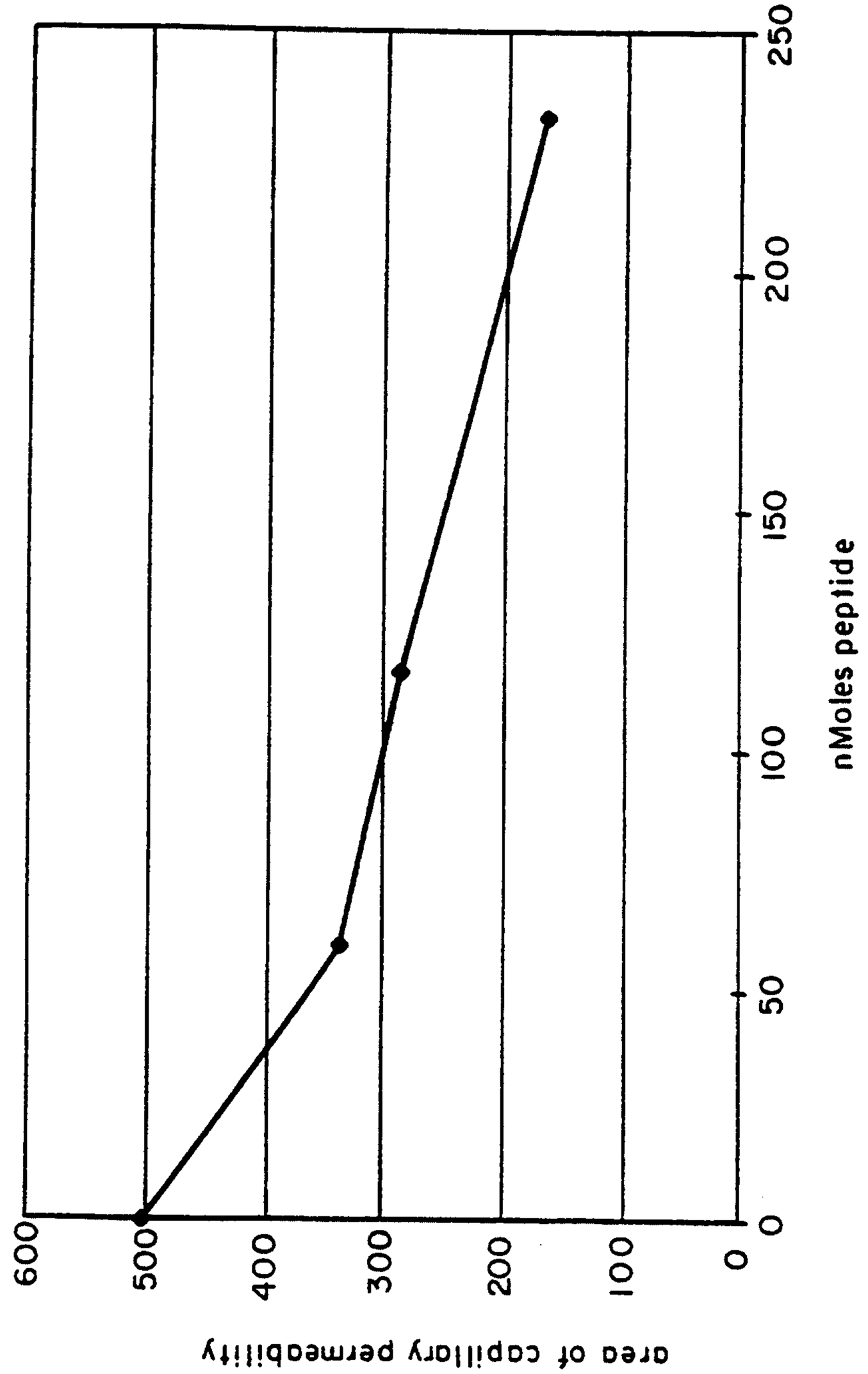
600
500
400
300
200
100
0
area of capillary permeability
0
50
100
150
200
250
nMoles peptide

FIG. 2

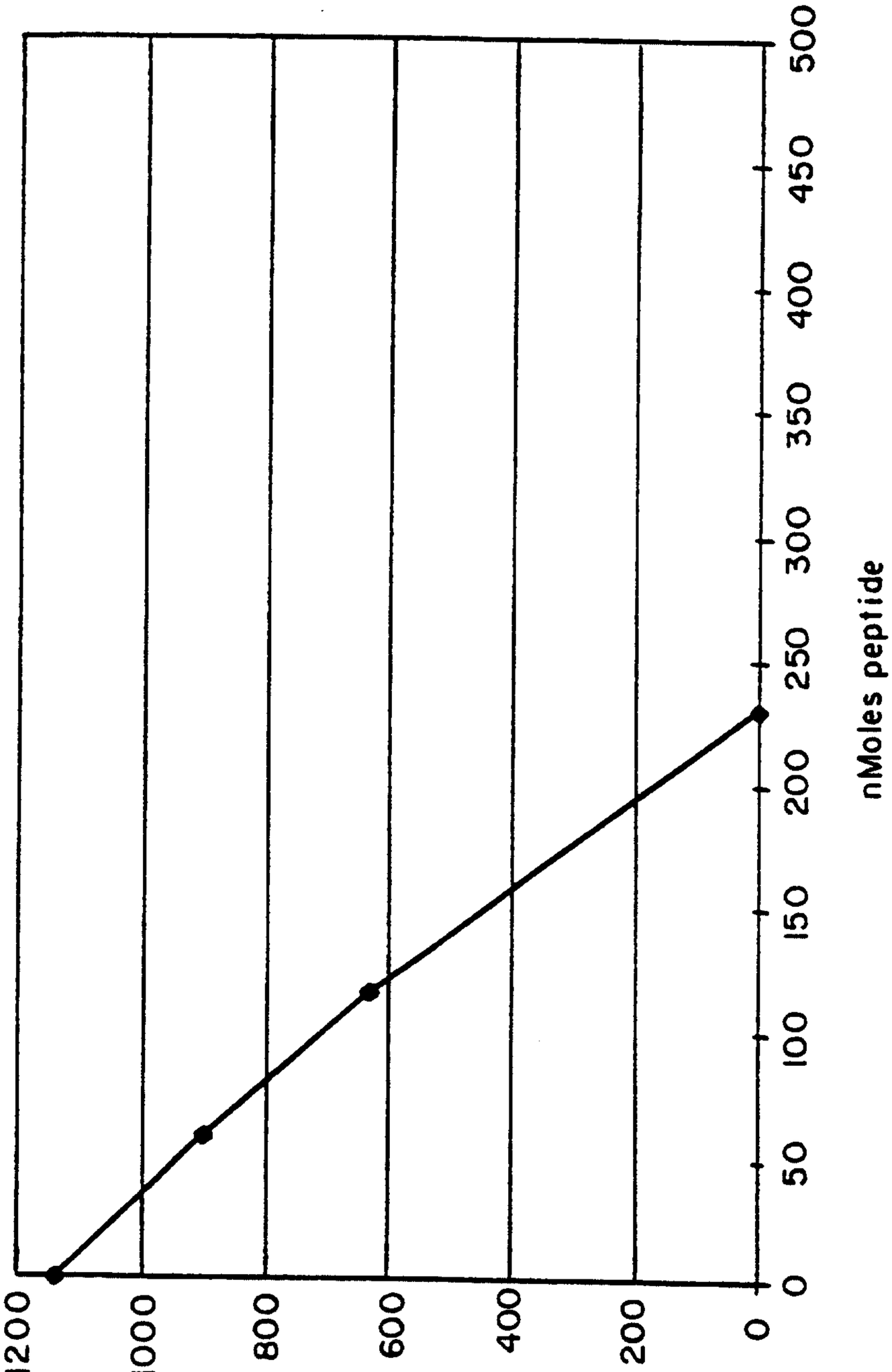
0
50
100
150
200
250
nMoles peptide
0
200
400
600
800
1000
1200
area of capillary permeability

FIG. 3

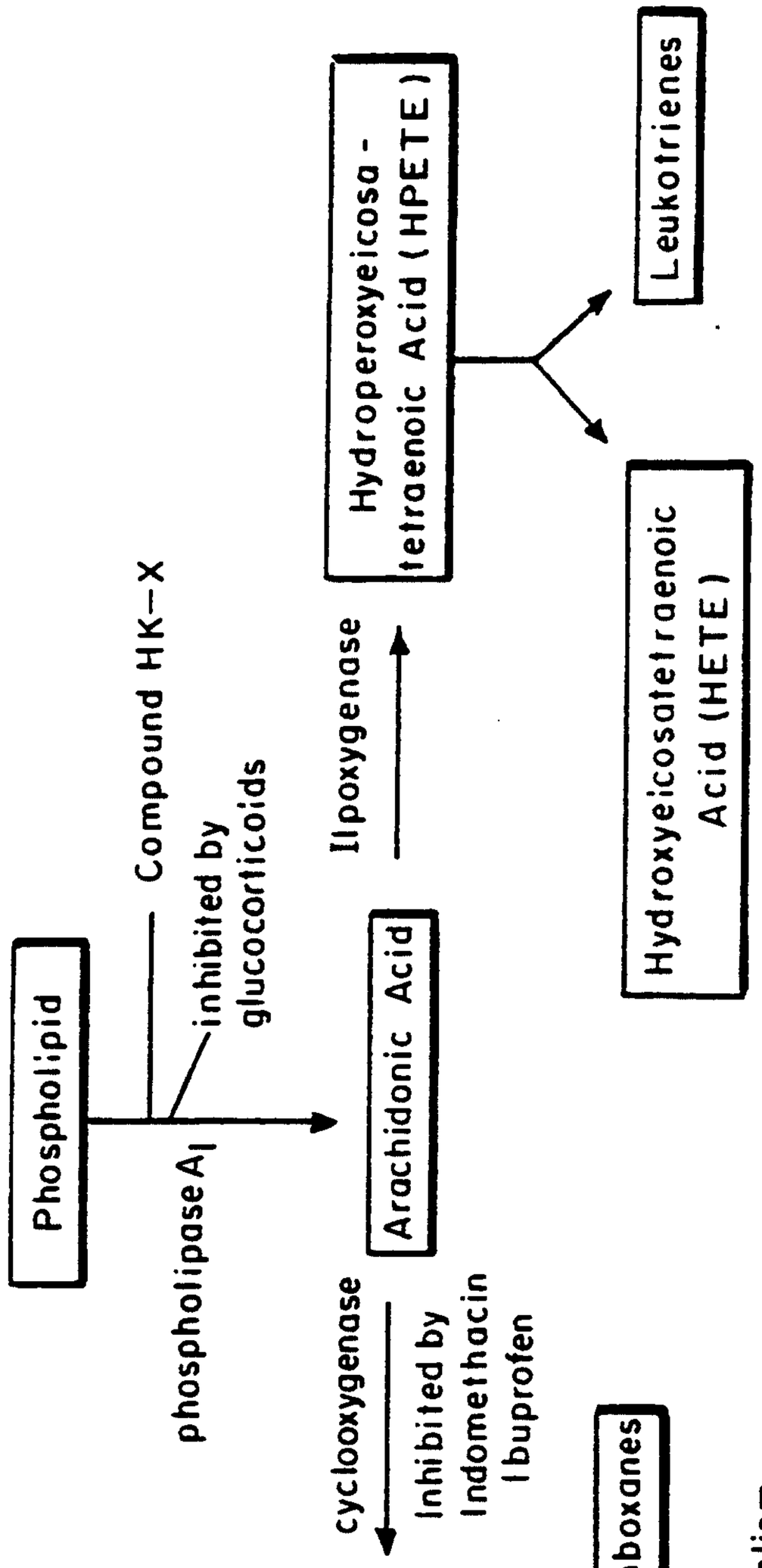
Phospholipid
phospholipase A1
Compound HK—X
inhibited by glucocorticoids
Arachidonic Acid
Ilpoxygenase
Hydroperoxyeicosa-tetraenoic Acid (HPETE)
Leukotrienes
Hydroxyeicosatetraenoic Acid (HETE)
cyclooxygenase
Inhibited by Indomethacin Ibuprofen
Cyclic Endoperoxides
Thromboxanes
Prostaglandins
Arachidonic Acid Metabolism

FIG. 4

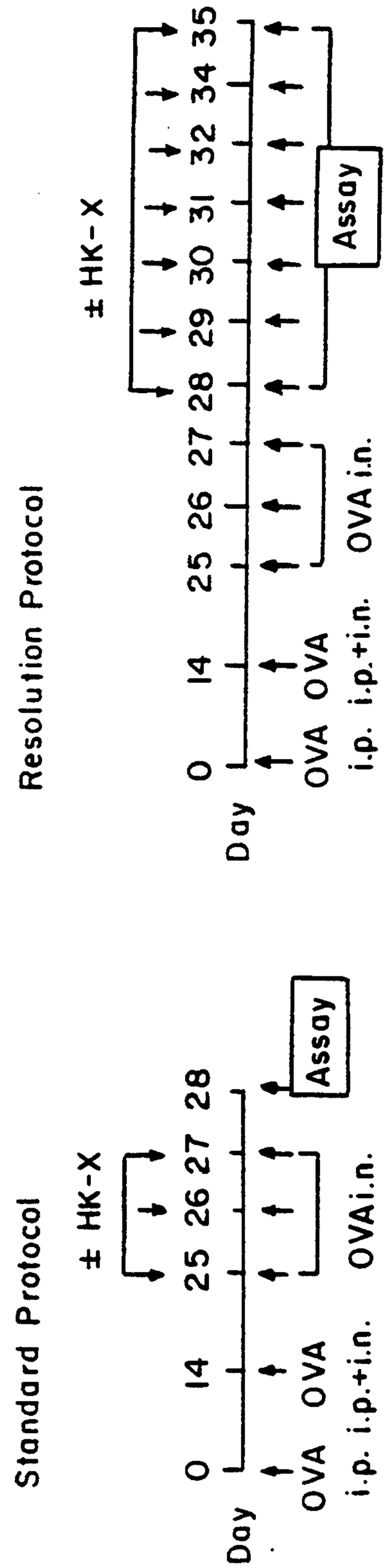
Standard Protocol
± HK-X
0
14
25 26 27 28
Day
OVA
OVA
i.p. i.p.+i.n.
OVAi.n.
Assay
Resolution Protocol
± HK-X
0
14
25 26 27 28 29 30 31 32 34 35
Day
OVA
OVA
i.p. i.p.+i.n.
OVA i.n.
Assay

FIG. 5A

FIG. 5B

FIG. 6A

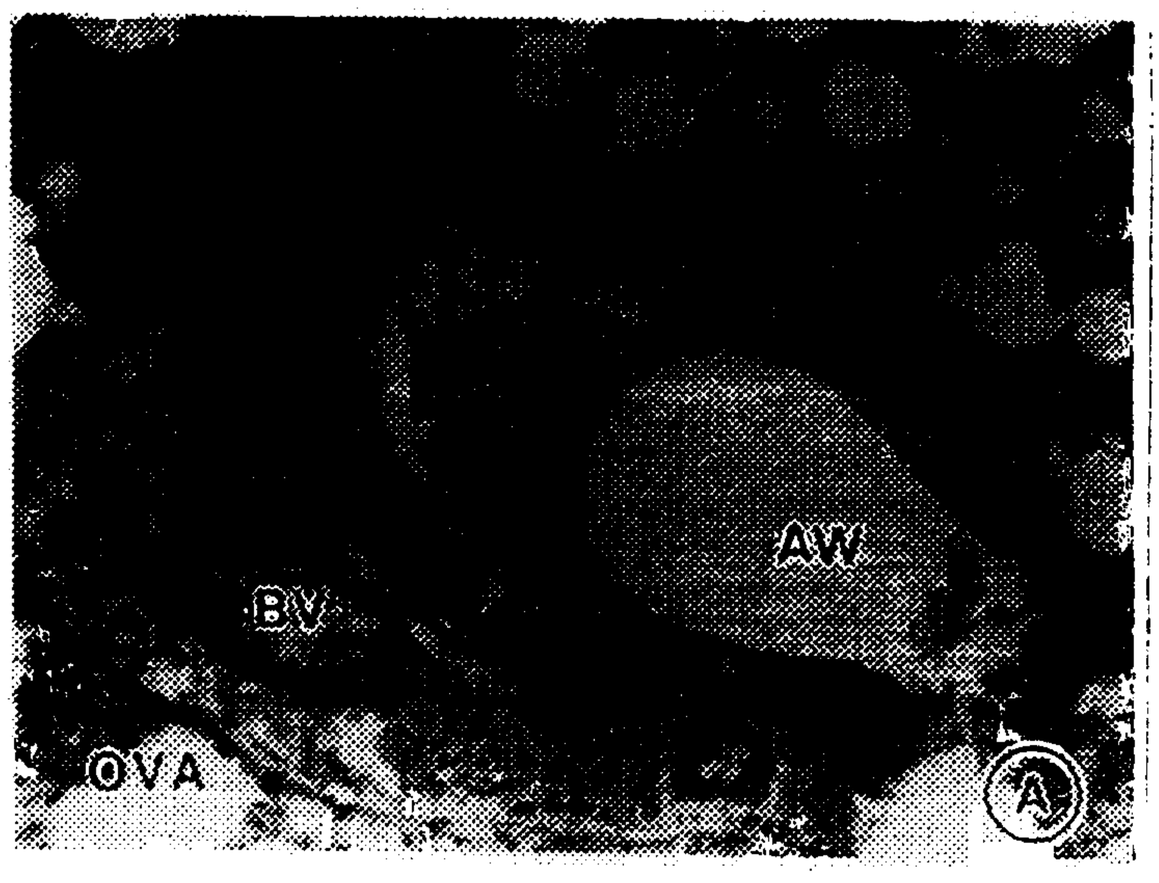

FIG. 6B



FIG.6C



FIG.6D



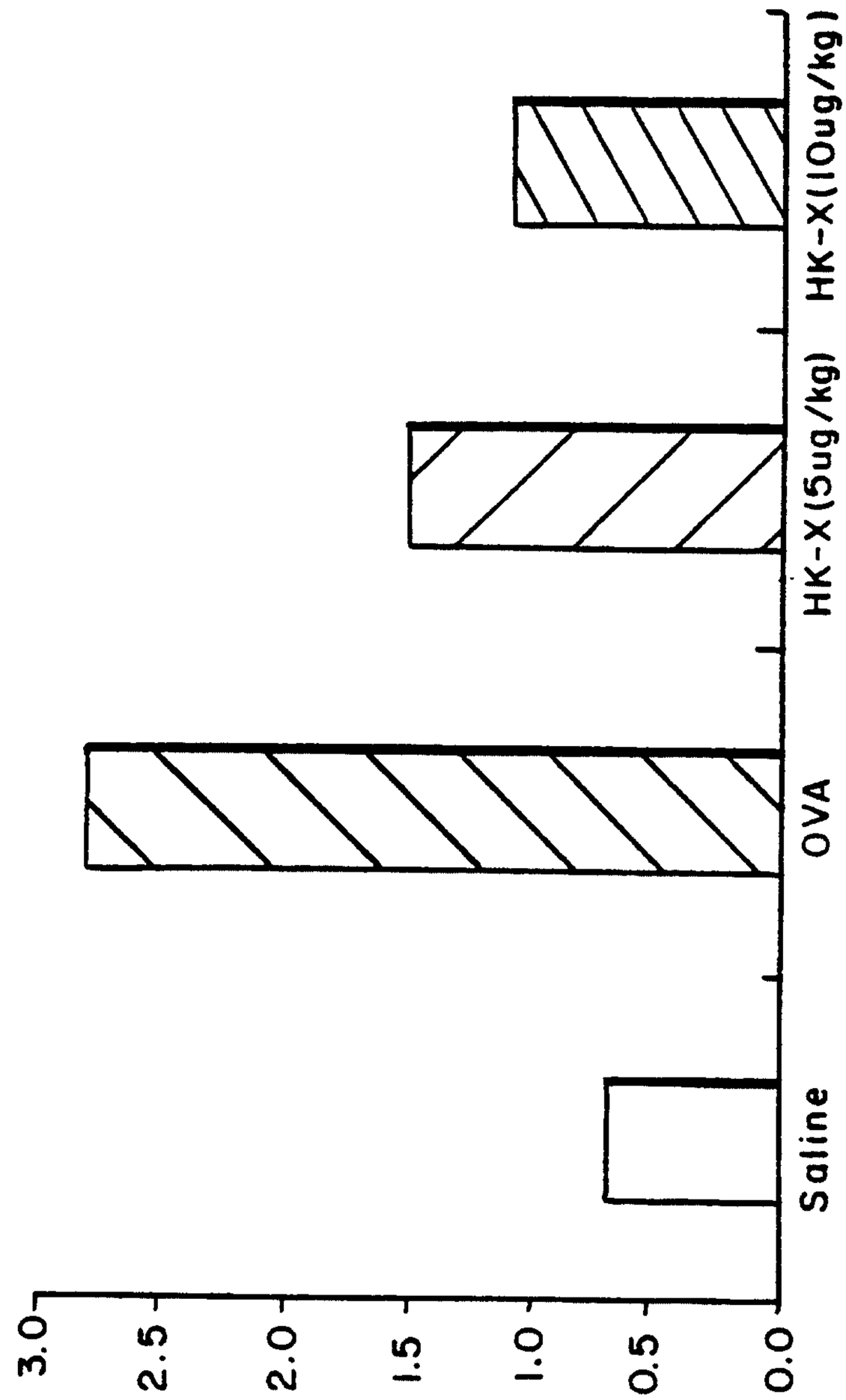
HK-X(10ug/kg)
HK-X(5ug/kg)
HK
OVA
Saline
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mucus plug score

FIG. 7