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VARIOUS ANIMAL MODELS FOR PRECLINICAL TESTING OF ANTI-INFLAMMATORY AGENTS

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ABSTRACT: Inflammation is the local defensive response of living tissues to injury or due to any other chemical agents. Inflammation consists of two basic processes early inflammatory response later followed by healing. There are two types of inflammation, acute and chronic. The different animal models (i.e. vascular permeability, UV-erythema in guinea pigs, Croton-oil ear edema in rats and mice, Paw edema in rats, Collagen Induced Arthritis, Adjuvant Induced Arthritis, and Papaya Latex Induced Arthritis) are used for evaluation of anti-inflammatory activity for pre clinical study. Animal models of inflammation are used to study pathogenesis of ailments and to evaluate potential anti-inflammatory drugs for clinical use. This review article addresses the inflammatory characteristics and similarities between varieties of animal models of humans.

INTRODUCTION: Inflammation is normal and necessary protective response to the harmful stimuli such as infectious agents, antigen-antibody reactions, thermal, chemical, physical agents, and ischemia [1, 2]. It is caused by a variety of stimuli, including physical damage, UV irradiation, microbial attack, and immune reactions. The classical key features of inflammation are redness, warmth, swelling, and pain. Inflammation cascades can lead to the development of diseases such as chronic asthma, arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis [3]. Inflammation is either acute, chronic and miscellaneous Inflammation [4, 5]. Acute inflammation may be an initial response of the body to harmful stimuli [6].

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Chronic inflammation starts in 2–4 days after the onset of the acute response and can last for weeks to months or years due to the persistence of the initiating stimulus, interference of the normal healing process, repeated bouts of acute inflammation, or low-grade soldering due to continued production of immune response mediators [7]. Chronic inflammation on the contrary is granuloma formation [8, 9]. Cyclooxygenase (COX) is the main enzymes in the procuction of prostacyclins, prostaglandins and thromboxanes which are involved in inflammation, pain and platelet aggregation [1, 6].

Methods for testing acute, sub acute and chronic inflammation:

- Vascular permeability
- UV-erythema in guinea pigs
- Croton-oil ear edema in rats and mice
- Oxazolone-induced ear edema in mice
- Pleurisy tests
- Paw edema in rats

- Granuloma pouch technique (various modifications and various irritants)

a. Cotton wool granuloma
b. Glass rod granuloma

- Collagen Induced Arthritis
- Adjuvant Induced Arthritis
- Papaya Latex Induced Arthritis

Vascular permeability: For the period of inflammation, vascular permeability increases to allow plasma components such as antibodies and harmonize to access infected or injured tissues. The test is used to estimate the inhibitory activity of drugs against increased vascular permeability which is induced by phlogistic substances. Inflammation is mediated by release of prostaglandins, histamine and leucotrienes following stimulation. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a result, plasma proteins and fluids are extravagated and edemas are produced. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan's blue [10].

Procedure:

- The Albino wistar rats are used each group containing 6 rats.
- Control group will receive distilled water one percent w/v one ml/100g by oral route and other group will receive test compound by oral route and standard group will get diclofenac 10ml/kg by intraperitoneal route.
- After one hour of these administration rats are injected with 0.25ml of 0.6 percent v/v solution of acetic acid intraperitoneally.
- Immediately, 10 ml/kg of ten percent w/v Evans blue is injected intravenously via tail vain. 5. After 30 minute, the animals are anesthetized with ether anaesthesia and sacrificed.
- The abdomen is cut open and exposed viscera. The animals are held by a flap of abdominal wall over a Petri dish. The peritoneal fluid collected, filtered and made up the volume to ten ml using normal saline solution and centrifuged at 3000 rotation per minute (RPM) for 15 minutes.
- The absorbance of the supernatant is measured at 590 nm using spectrophotometer.

Assessment: The concentration of dye decreased with respected to absorbance indicates decrease in permeability. The assessment result of test group is compared with that of standard group. ED_{50} values can also be calculated [11].

UV-Erythema in Guinea Pigs: Prostaglandin E (PGE) levels in the skin have been revealed to be increased during the 24 hours period subsequent exposure of guinea pig skin to UV (ultraviolet radiation) from 280-320 nm. The progress of increased PGE levels paralleled the development of the delayed phase of erythema. Delay the development of ultraviolet erythema on albino guinea pig skin by systemic pre-treatment with clinically comparable doses of phenylbutazone and other NSAIDs (Nons-teroidal Anti-Inflammatory Drugs). Redness (erythema) is the initial sign of inflammation, not yet accompanied by plasma exudation and edema [12].

Procedure:

- The Albino guinea pigs of both sexes with an average weight of 350g are used.
- Six animals are used each for treatment and control group.18 hr prior testing, the animals are shaved on both the flanks and on the back.
- After that, they are chemically depilated by a by a suspension of barium sulphide. 20 minutes later, the depilation paste and the fur are rinsed off in running warm water.
- Next day the trial compound is dissolved in the vehicle and 1/2 of the test compound is administered by gavages (at 10 ml/kg) 30 minutes before UV contact.
- Control animals are treated with the vehicle alone.
- The guinea pigs are placed in a leather cuff with a hole of 1.5×2.5 cm size punched in it, allowing the UV radiation to reach only this area. Then animals are exposed to UV

radiation. 7. After 2 minutes of expose the remaining half of the test compound is administered. The erythema is scored 2 and 4 hours after exposure.

Assessment: The degree of erythema is evaluated visually by 2 different investigators in a double blinded manner. The scores are given: 0 = No erythema, 1 = Weak erythema, 2 = Strong erythema, 4 = Very strong erythema.

Animals with a score of 0 or 1 are considered to be protected. The scoring after 2 and after 4 h gives some indication of the duration of the effect. *ED*50 values can be calculated [11, 13, 14].

Croton-oil ear edema in rats and mice: It contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. TPA is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen activated protein kinases (MAPK), and phospholipase A2 (PLA2), leading to release of platelet activation factor (PAF).

This flow of events stimulates vasodilation, vascular permeability, polymorphonuclear leukocytes migration, release of histamine and serotonin and moderate synthesis of inflammatory eicosanoids by 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) enzymes. COX and 5-LOX inhibitors, leukotriene B4 (LTB4) antagonists and corticosteroids showed topical anti-inflammatory action in animal models of Croton oil or TPA induced skin inflammation [15, 16].

Procedure:

- Six animals are used each for treatment and control group.
- The total of 15µl of an acetone solution containing 75µgm of croton oil is applied to the inner surface of right ear of each mouse. Left ear remains untreated.
- Control animals receive only the irritant while indomethacin (100µg/ear) serves as reference.
- Diffrent dose levels of test compounds are applied to the inner surface of right ear of each mouse by dissolving them in inflammation inducing solution.
- Animals are sacrificed by cervical dislocation after 6 hours and a plug (6 mm in diameter) is removed from both the treated and untreated ear.
- The difference in weight between the two plugs is taken as measure of edematous response. Since tetradecanoyl porbol acetate (TPA) is the chief ingredient of croton oil, purified TPA has also been used to induce ear edema in mice.

Assessment: The antiphlogistic effect can be evaluated by expressing the increase in weight of the treated ear as percentage of the weight of the contra lateral control ear. The difference between both ears and excised discs is calculated as the average values for treated and control groups and the effect is evaluated by statistical methods [17-19].

Oxazolone-induced ear edema in mice: It is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration. The oxazolone-repeated challenge increased the level of Th2 cytokines and decreased that of a Th1 cytokine in the lesioned skin. The Th2 cytokines, especially IL-4, play major roles in the development of dermatitis in the present mouse model [20].

Procedure:

- Each contains 12 mice, the same skin site of the right ear was sensitized by a single application of 10 µl (each 5 µl for inner and outer of ear) of 0.5% oxazolone in acetone 7 days before the first challenge (day 0), and 10 µl of 0.5% oxazolone in acetone was repeatedly applied to the sensitized right ear 3 times per week.
- The only acetone will be applied to the right ear. The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2% oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is solved.
- One group of animals are treated with the irritant alone or with the solution of the test

compound. The left ear remains untreated. The maximum of inflammation after 24 hour.

- At this stage the animals are sacrificed under anaesthesia and a disc of 8 mm diameter is punched from both sides. The discs are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema.

Assessment: The mean of average values of the increase of weight are calculated for every test and control group compared statistically [21, 22].

Pleurisy tests: Pleurisy can be induced in animals by various irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan [23].

Procedure: The mouse pleurisy was induced by a single intrapleural injection of 0.1 ml of carrageenan (1%). After 4 h the animals were killed with an overdose of ether, the thorax was opened and the pleural cavity was washed with 1.0 ml of sterile PBS, containing heparin (20 IU per ml). Samples of the pleural lavage were collected for determination of exudation, myeloperoxidase, adenosine-deaminase activities, and nitric oxide levels, as well as for determination of total and differential leukocyte counts. Total leukocyte counts were performed in a Neubauer chamber.

The cytospin preparations of pleural wash were stained with May–Grunwald Giemsa for the differential count which was performed under an oil immersion objective. The serum level of the Creative protein was also analysed. In another set of experiment animals were treated 30 min before carrageenan with a solution of Evans blue dye (25mg/kg, i.v.) in order to evaluate the degree of exudation in the pleural space. A sample (500 µl) of the fluid leakage collected from the pleural cavity was stored in a freezer (−20 °C) to further determine the concentration of Evans blue dye. To this end, on the day of the experiments, a batch of samples was thawed at room temperature and the amount of dye was estimated by colorimetry using an Elisa plate reader at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.0 to 50 µg/ml.

Evaluation: One ml (the added Hank's solution) is subtracted from the measured volume. The values of each experimental group are averaged and compared with the control group. *ED*50 values can be calculated using various doses [24, 25].

Paw edema in rats: The ability of anti-inflammatory drugs to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil etc. The volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Carrageenan-induced rat paw edema is associated with three distinct phases. The first phase is early mediated by mast cell degranulation and histamine and serotonin release (1 h), the second phase (60 to 150 min) is characterized by bradykinin release and pain, and further eicosanoid production in the late phase (3-4 h). So here the anti-inflammatory effect of the test compound is due to inhibition of which mediator can also be known.

Procedure: Male or female Sprague-Dawley rats with a body weight between 100 and 150 g are used. The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6h, and eventually 24h after challenge. Various devices have been developed for plethysmography of the paw, like mercury for immersion of the paw, more sophisticated apparatus based on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer, sensitive method of measuring mouse paw volume by interfacing a Mettler Delta Range top-loading balance with a microcomputer, commercially available plethysmometer.

Evaluation: The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated. The difference at the various time intervals gives some hints for the duration of the anti-inflammatory effect. A dose- response curve is run for active drugs and *ED*50 values can be determined [11, 19].

Granuloma pouch technique: With the introduction of an irritant substance into an s.c. air pocket, granulation tissue begins to proliferate and soon covers the whole inside of the pouch. This tissue consists of fibroblasts, endothelial cells and an infiltrate of macrophages and polymorph nuclear leukocytes. In the GPA this rapidly growing tissue can be exposed to carcinogenic and mutagenic substances. One of the major advantages of the system is the possibility of bringing the test compounds into direct contact with the target cells, by injecting them into the air pocket. It is also possible to administer the material by the oral and parenteral routes. It does not provide quantitative information on cyto-toxicity of the test compounds *in vivo*.

Procedure: Male or female Sprague-Dawley rats with a body weight between 150 and 200 g are used. Ten animals are taken for controls and for test groups. The back of the animals is shaved and disinfected. With a very thin needle a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anaesthesia. Into the resulting oval air pouch 0.5 ml of a 1% solution of Croton oil in sesame oil is injected avoiding any leakage of air.

Forty-eight hours later the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken. Instead of croton oil 1 ml of a 20% suspension of carrageenan in sesame oil can be used as irritant. Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard. For testing local activity, the test compound is injected directly into the air sac at the same time as the irritant. On the 4th or the 5th day the animals are sacrificed under anaesthesia. The pouch is opened and the exudate is collected in glass cylinders. The pouches are washed with 1 ml of saline, exudates are immediately cooled on ice and the volume is recorded. Total of leukocytes migrated into the pouch are evaluated after staining with Erythrosine B and remaining exudates is centrifuged at 3000 rpm for 10 min at 4 degrees and supernatant stored at -20 degrees until use.

Evaluation: The average value of the exudates of the controls and the test groups is calculated. Comparison is made by statistical means [26-27].

Methods to evaluate proliferative phase of inflammation:

Cotton wool granuloma: The foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histological giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenin.

Procedure: Male rats weighing 180–200 g were used. Test drugs were administered orally on a once daily dosage regimen for 7 days, and the control group received vehicle. Two sterilized pellets of cotton wool were implanted subcutaneously, one on each side of abdomen of the animal, under the light ether anaesthesia and sterile technique. The rats were sacrificed on the eighth day. The implanted pellets were dissected out and recorded for wet weight. Thymuses were also dissected out. Both pellet and thymus were dried at 60°C for 18 h and the dry weight was recorded.

Evaluation: The weights of the transudate and the granuloma as well as the percent granuloma inhibition of the test drugs were calculated. The body weight gain was also recorded [19, 28].

Glass Rod Granuloma: These reflect the chronic proliferative inflammation. Of the newly formed connective tissue not only wet and dry weight, but also chemical composition and chemical properties can be measured.

Procedure: Glass rods with a diameter of 6 mm are cut to a length of 40 mm and the ends rounded off by flame melting. Male Sprague-Dawley rats with an initial weight of 130 g are anaesthetized with ether, the back skin shaved and disinfected. From an incision in the caudal region a subcutaneous tunnel is formed in cranial direction with a closed blunted forceps. One glass rod is introduced into this tunnel finally lying on the back of the animal. The incision wound is closed by sutures. The animals are kept in separate cages. The rods remain *in situ* for 20 or 40 days. Treatment with drugs is either during the whole period or only during the last 10 or 2 days. At the end the animals are sacrificed under CO_2 anaesthesia.

The glass rods are prepared together with the surrounding connective tissue which forms a tube around the glass rod. By incision at one end the glass rod is extracted and the granuloma sac inverted forming a plain piece of pure connective tissue. Wet weight of the granuloma tissue is recorded. Finally, the granuloma tissue is dried and the dry weight is recorded. In addition, biochemical analyses, such as determination of collagen and glycosaminoglycans can be performed.

Evaluation: Several parameters can be determined by this method. Granuloma weight was reduced by test compound is compared with that of standard [11, 29].

Collagen-Induced Arthritis (CIA): Collagen Induce Arthritis is induced by intradermal injections of CII together with an adjuvant. Most commonly, heterologous CII of chick, bovine or rat origin is used to induce the disease. CIA is Major Histocompatibility Complex (MHC) dependent and is characterized by erosive joint inflammation mediated by both T and B cells. The pathogenesis and disease course vary noticeable, though, depending on the genetic background of the mice and on the origin of the CII used for disease induction [30, 31].

Procedure:

- Experimental mice will be 8-10 weeks old when immunized. Native type II collagen was dissolved in 0.1 N acetic acid at a concentration of 2mg/ml by stirring overnight at 4°C.
- The heat killed *Mycobacterium tuberculosis* was ground with a mortar and pestle and added to Incomplete Freund's adjuvant (IFA). Equal volumes of adjuvant and collagen solution were emulsified for two minutes at high speed with a homogenizer.
- Each mouse received intradermal injection of 0.1 ml of the resulting emulsion in the tail.
- Other groups of mice were immunized in the same manner with 100 µg of denatured CII. 5. The booster injection of 100 µg of antigen emulsified in Incomplete Freund's adjuvant was given by intradermal injection in the dorsal skin on day 28.
- The control group will be received an equal volume of 0.1 N acetic acid emulsified with IFA and containing 250 µg of *Mycobacterium tuberculosis* as a primary injection, and acetic acid in IFA alone as a booster on day 28.

Assessment: The arthritic index will be assigned to each mouse by using the following criteria: 0, no signs of arthritis; 1, swelling and redness in a single joint; 2, inflammation in multiple joints; and 3, severe swelling, joint erosion, and/or ankylosis. Each paw was scored from 0–3, and the arthritic paws then were multiplied by their score with the index being the sum of all of the paws [32, 33].

Adjuvant-induced arthritis model in rats: The objective of the rat adjuvant-induced arthritis model was focused on the eicosanoid pathway and in particular on inhibitors of cyclooxygenases (COX 1 and COX2)—the NSAIDs (non-steroidal anti-inflammatory drugs) [34].

Procedure:

- Arthritis was induced by a single intra-dermal injection (0.1 ml) of Freund's Complete Adjuvant (FCA) containing 1.0 mg dry heat-killed *Mycobacterium tuberculosis* per millilitre sterile paraffin oil into a foot pad of the left hind paw of male rats.
- A glass syringe (1 ml) with the locking hubs and a 26G needle was used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant Injection, as the

very viscous nature of the adjuvant exerts difficulty while injecting.

- The swelling paws were periodically examined (up to 21 days) in each paw from the ankle using digital plethysmometer [35-37].

Papaya latex induced arthritis: Papaya latex induced model of experimental rheumatoid arthritis has been developed to test the anti-inflammatory activity of slow reaction anti-rheumatic drugs (SARDs). Papaya latex induced inflammation is known to be mediated through prostaglandins.

Procedure:

- Prepare 0.25 % solution of papaya latex take 0.1ml (prepared in 0.05M sodium acetate buffer, pH 4.5 containing 0.01% thymol) is injected into the rat hind paw.

- The peak effect occurs at 3 hrs and lasts for more than 5 hrs. The method is sensitive for evaluating NSAIDs like aspirin, ibuprofen and steroidal anti-inflammatory drugs (SAIDs) [38-40].

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