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Various Screening Methods for Anti- Asthmatic Activity

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Abstract: Asthma is defined as a complex allergic disease of respiratory system which is characterized by airway inflammation & bronchial hyper-responsiveness to many allergens and due to interaction of many components of immunity system & resident lung cells. Asthma is currently a worldwide problem with around 300 million people around the globe suffering from it and about 250 thousand deaths are reported annually. Asthma remains an area of considerable unmet medical need. Few new drugs have made it to the clinic during the past 50 years, with many that perform well in preclinical animal models of asthma, failing in humans owing to lack of safety and efficacy. The utility of *in vivo* studies and demand for more predictive models and tools are based on the latest technologies. Studies have been conducted with *in vitro* and *in vivo* models but disease mechanism are still unclear. At present, there are no standardized experimental protocol models for asthma, most laboratories have developed and adopted own protocol with little modification. In this article, we discuss various *in vivo*, *in vitro* models of asthma which may describe the various mechanisms & patho-physiology involved in allergic and inflammatory disease & also provide investigation of new therapeutic substances for allergic disorders.

Keywords: Anti-asthmatic models, Clinical Research, Siddha medicine.

1. INTRODUCTION

Asthma is a reversible obstructive disease of the lower airway. With asthma there is increasing airway obstruction caused by bronchospasm and bronchoconstriction, inflammation and edema of the lining of the bronchioles, and the production of thick mucus that can plug the airway [1].

There are three types of asthma: [2]

1) Extrinsic (also referred to as allergic asthma and caused in response to an allergen such as pollen, dust, and animal dander).

2) Intrinsic asthma (also called non-allergic asthma and caused by chronic or recurrent respiratory infections, emotional upset, and exercise).

3) Mixed asthma (caused by both intrinsic and extrinsic factors).

Extrinsic or allergic asthma causes the IgE inflammatory response [3] with exposure; the IgE antibodies are produced and attached to mast cells in the lung. Re-exposure to the antigen causes them to bind to the IgE antibody, releasing histamine and other mast cell products [4]. The release of these products causes bronchospasm, mucous membrane swelling, and excessive mucous production. Gas exchange is impaired, causing carbon dioxide to be trapped in the alveoli so that oxygen is unable to enter [5].

Pathophysiology of Asthma

In asthma, smooth muscle that surrounds the bronchi is hyper responsive to stimuli, and underlying inflammatory changes are present in

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the airways. Asthmatic stimuli include inhaled allergens, occupational allergens, and drugs or non-specific stimuli, such as cold air, exercise, stress and pollution [6]. The stimuli cause asthmatic changes through several complex pathways. The possible mechanisms of these pathways include the following: [7].

Immune reactions (type1hypersensitivity) and release of inflammatory mediators – the cross-linking of IgE by allergens causes mast cell degranulation, and release of histamine and powerful eosinophil and neutrophil chemotactic factors [8]. The mediators, viz. histamine, tryptase, LTC4 and D4, and PGD2, when released enter through airway mucosa and stimulate mucosa and stimulate muscle contraction and vascular leakage, i.e. early asthmatic response. Re-exposure to allergen causes the synthesis and release of a variety of cytokines, viz. interleukin-4 (IL4) and IL5, granulocyte -macrophase colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF), and tissue growth factor (TGF) from T cell and mast cells [9]. These cytokines attract and activate eosinophils and neutrophils, which re-create eosinophil cationic protein, proteases, and platelet activating factor (PAF). These mediators cause edema, mucous hyper secretion, bronchoconstric-tion, and increase in bronchial activity associated with late asthmatic response [10].

An imbalance in airway smooth muscle tone involving the parasympathetic nerves (vagus), non-adrenergic non-cholinergic nerves and circulating noradrenalin that acts under normal circumstances to control airway diameter.

Abnormal calcium flux across cell membranes, increasing smooth muscle contraction and must cell degranulation.

Leaky tight junctions between bronchial epithelial cells allowing allergen access.

EXPERIMENTAL MODELS FOR ASTHMA

Experimental Protocols

At present there is no standardized experimental protocol for asthma. Most laboratories developed their own protocols either with major or minor modifications according to their needs. Depending on a single model may possess many problems when data are analysed. Similarly, when number of models are used, it is very difficult to compare the results from different studies. As the experimental approach is so important to the outcomes, it is an area ripe for consolidation and standardization.

Paw Oedema (Histamine, Serotonin and Bradykinin)

The rats were divided into 5 groups as follows: Group I received saline, Group 2 received the reference drug indomethacin 10mg/kg and Groups 3-5 received investigational drug at 100, 300 and 500mg/kg respectively. The drugs were orally administered to the respective groups of 5 rats each (N=5). After one hour, paw oedema was induced in the rats by subcutaneous injection of 0.1ml of 1% histamine and serotonin into the sub-plantar of the experiment rats and the paw volume was measured with a plethysmometer at 0hr, 1hr, 2hrs, 3hrs, 4hrs and 5hrs after the injection of the mediators. The procedure was repeated for bradykinin, but bradykinin was injected alongside with perindopril arginine and paw volume was measured at 0, 10, 20, 40 and 60 minutes [11]. The inhibitory activity was calculated according equation [12].

Histamine and Acetylcholine Induced Broncho-constriction

The guinea pigs were divided into 5 groups: Group I received saline, Group 2 received pyrelamine maleate and Groups 3-5 received Investigational drug at concentration of 100, 300 and 500mg/kg. The drugs were orally administered to the respective groups of 5 rats each (N=5). After overnight fasting of the rats, they were exposed to 0.2% histamine and acetylcholine aerosol in an air-tight chamber and the pre-convulsion time (PCT) (time of aerosol exposure to the onset of dyspnoea leading to appearance of convulsion) was recorded and they were immediately removed from the chamber and placed in fresh air to recover. The rats were left for 24 hours to recover and were given their various treatments according to the grouping above and were again exposed to histamine and acetylcholine aerosol at1hr, 4hrs and 24hr after treatment and their PCT was again recorded this was PCT after the administration of the drugs [19].

Percentage protection = [1 – (T1 / T2)] x 100

Histamine, Acetylcholine, Serotonin and Bradykinin Induced Contraction in GPI

The abdomen of the guinea pig was opened and the part of the ileum nearest to the caecum was removed and placed in a beaker filled with aerated Krebs-Henseleit solution(in mM): NaCl, 118.41; KCl, 4.69; $NaHCO_3$, 25.0; $CaCl_2.2H_2O$, 2.52; $MgSO_4.7H_2O$, 1.22; KH2PO4, 1.18; glucose anhydrous, 9.991) [13]. 1cm Ileum (1cm) was cut off and placed in a petri dish containing aerated Krebs-Henseleit solution and the residues left in the luminal area were carefully washed off with Krebs- Henseleit solution with the aid of an oral feeding gauge.

After washing, the tissue was suspended in a 50 ml organ bath filled with aerated Krebs-Henseleitsolution with one end of the tissue attached to a tissue holder and the other end to an isometric force transducer using a waved cotton thread. This preparation was incubated for 30-60 minutes during which the bathing solution was changed every 15 minutes. The tissue was maintained at 1 g tension [14]. The contraction induced by histamine, acetylcholine, serotonin and bradykinin was challenged with 100ug and 500ug of Investigational drug in the organ bath [15]. The concentration –response curves were recorded.

Evaluation of Antiasthmatic Activity: Solated Guinea Pig Ileum Preparation [16-19]

Guinea pigs of either sex (250-400 gm) were grouped into four. Each group contains six animals and were allowed to starve overnight and given free access to water. Animals were sacrificed by cervical dislocation and carotid bleeding. The trachea was dissected out and transferred to a dish containing kerb's solution (composition (g/l): NaCl (6.5), KCl (0.33), $CaCl_2$ (0.26), $MgSO_47H_2O$ (0.28), $NaHCO_3$ (2.5), KH_2PO_4 (0.19) and glucose 5.0) and cut crosswise between the sections of the cartilage of the trachea and continuously ventilated and maintained at 37 ± 1°C. The adjourned trachea was allowed to make steady for at least 40 minutes. On equilibrium, the bath was supplied with fresh kerb's solution for every 15 minutes. Then cumulative concentration response to histamine in the absence and presence of investigational drug were recorded with a (0.25 mm/sec) kymograph [20-22].

Histamine Aerosol Induced Bronchoconstriction in Guinea Pigs [23-25]

Histamine was dissolved in distilled water to prepare 0.2% w/v solution. Experimentally bronchial asthma was induced in guinea pigs by exposing histamine aerosol by an ultra-sound nebulizer in an aerosol chamber (30 x 15 x 15cm) made of Perspex glass. The required time for appearance of pre-convulsive dyspnoea produced by the histamine was noted for each animal. Each animal was placed in the histamine chamber and exposed to 0.2 % histamine aerosol. The pre-convulsion time (PCT), i.e. the time of aerosol exposure to the start of dyspnoea leading to the appearance of convulsion, was noted. As quickly as the pre-convulsion dyspnoea (PCD) was recorded, the animals were removed from the chamber and positioned in fresh air forrecover.

This time for pre-convulsive dyspnoea was recorded as basal value. Guinea pigs were then allowed to recover from dyspnoea for 2 days. After that, the animals were allotted to four different groups of 4-5 animals per group. Animals in group 1 served as control and received distilled water. The animals of group 2 and 3 were given, by oral intubation, 200 and 500mg/kg of the Investigational drug, respectively, while group 4 received the standard drug –Chlorpheniramine maleate, intra-peritoneally. After receiving the drugs, all the animals were again exposed to histamine aerosol in the chamber, one hour, four hours and 24 hrs, to determine pre convulsive time (PCT). The protection untaken by the treatment was calculated using the formula:

Percentage Protection

Eta -Etb ×100(% Protection offered by the extract)

ETB:

Where: Eta is the mean of PCT (Preconvulsion time) before administration of test drugs. Etb is the mean of PCT (Preconvulsion time) after administration of test drugs at 1 hr, 4 hr and 24 hrs.

Anti-asthmatic Activities

Albino guinea pigs (350 to 400 g) and Albino rats (160 to 200 g) of either sex were used for the experiments. They were kept in standardized

environmental conditions and maintained on a standard diet and water ad libitum and starved over night before the experiments. For anti-asthmatic activity, guinea pigs were sensitized with three doses of egg albumin (200 mg/kg) intra-peritoneally as well as subcutaneously. The egg albumin doses were given on 1st, 3rd and 5th days. Three weeks after the last dose, the animals were utilized for the experiments and effects of test drugs were investigated on egg albumin challenged guinea pig and their isolated tracheal strip or ileum [26, 27].

For anti- passive cutaneous anaphylaxis (PCA) activity, rats were injected intramuscularly with 0.5 ml of a physiological saline solution containing 10 mg egg albumin. In addition, Bordetella pertussis vaccine (0.2 ml containing 1.8 x 109 cells) was injected intraperitoneally to each rat on 1st, 3rd, and 5th days. Four weeks after the first immunization, the blood was collected from heart and serum containing IgE type antibodies was separated out by cooling centrifugation and stored at -8 ± 10 C and utilized for the experiments [28, 29].

Bronchodilator Activities on Different Models Isolated Tracheal Strip of Guinea pigs

The guinea pigs were killed by exsanguinations and trachea was dissected out and cut into strips. The tracheal preparation was mounted in an organ bath maintained at 37±0.50 C, containing Krebs physiological solution and constantly gassed with air. The isolated tracheal strip was connected to force displacement transducer (Bio-Device, India). All tracheal preparations were allowed 1 hour for equilibration before testing of compounds or standard (Aminophyline). The contraction responses of standard agonist like acetylcholine (4.42 x 10-5 M), histamine (4.08 x 10-5 M) and egg albumin, 200 µg/ml. of bath concentration (only in sensitized tissue) on tracheal strip were recorded on student physiograph [30].

The same responses were re-elicited in the presence of compounds or aminophyline at different concentrations.

In another set of experimental investigation, a sustained contraction of the tracheal strip was elicited using carbamylcholine chloride (4.10 x 10-5 M). After 10 minutes, the tracheal strip was exposed to different concentrations of compounds or aminophyline (5.95 x 10-4 to 1.78 x 10 -3 M) [31] and the relaxation in tracheal strip was recorded on student physiograph (Bio- devices, India).

Broncho-protective Activity in Guinea pigs Against Various Mediators

Experimental bronchospasm was induced in guinea pigs by exposing them to either 4 % w/v acetylcholine bromide in 5 % NaH2PO4 or 2 % w/v histamine dihydrochloride or 5 % egg-albumin (in sensitized guinea pigs) aerosol at a constant pressure using histamine chamber. The guinea pigs under exposure to acetylcholine or histamine or egg- albumin shown progressive dyspnea. The end point of pre- convulsive dyspnea (PCD) was determined from the time of aerosol exposure to the onset of dyspnea leading to the appearance of convulsion. As soon as PCD commenced, the animals were removed from the chamber and placed in fresh air for recovery [32]. After two days interval the same group of guinea pigs was administered with different doses of compounds or aminophylineintraperitoneally, 45 min. after the drug administration, the time for the onset of PCD was recorded against the experimental bronchospasm induced by acetylcholine or histamine or egg-albumin aerosol [33]. The protection offered by different drug treatment was calculated by following formula [34]. Percentage protection = [1-(T1 / T2)] X 100, Where T1 was a mean of control PCD time and T2 was a PCD time after drug treatment.

Experiments on Sensitized Guinea pig Using Various Mediators

Sensitized guinea pigs were killed by exsanguinations. The ileum was dissected out and cut into small pieces (2 cm. long) and connected to force displacement transducer (Bio-devices, India) in a 20 ml. capacity of organ bath containing Tyrode solution, maintained at 37±0.50 C and gassed with air. All isolated ileum preparations were allowed 1 hour for equilibration before the testing of the drugs [35]. The contraction responses of standard agonist like acetylcholine (3.75 x 10 -7 M), histamine (2.71 x 10 -7 M), egg albumin (100 µg/ ml) and barium chloride (2.05 x 10 -3 M) on isolated ileum were recorded on student physiography [36, 37]. The same

responses were re-elicited in the presence of compounds or aminophyline at different concentrations.

Mast Cell Stabilizing Activity on Rat Mesentery

Albino rats were killed by exsanguinations. The piece of small intestine along with mesentery of rat was excised and connecting lobes of fat and blood vessels were rapidly dissected out. The piece of mesenteries was spread on petri-dish containing Ringer-Locke physiological solution [38]. Compounds at different concentration on standard disodium cromoglycate incubated mesenteries were challenged with 1 µg/ ml of compound 48/80 (standard granulator) for 10 minutes [39]. Pieces of mesentery were stained with 0.1 % toluidine blue solution containing 4% formaldehyde for 20 to 30 minutes and then treated through acetone and xylene and mounted on slides [40]. The percentage of degranulated mast cells was counted under microscope and protection offered by various drugs treatment was calculated.

Experiments on Homologous PCA in Rats

The homologous anti- PCA technique was used [41]. The rats were injected intramuscularly with 0.5 ml. of a physiological saline solution containing 10 mg egg-albumin. In addition, Bordetella pertussis vaccine (0.2 ml containing 1.8 x 109 cells) is injected intraperitoneally to each rat on 1st, 3rd and 5th day. Four weeks after the first immunization, the blood was collected from heart and serum containing IgE type antibodies, was separated out by cooling centrifugation and stored at -8 ± 10 C, till being used for homologous PCA testing. The serum containing IgE antibodies was injected intradermally to rats at four shaved sites (0.1 ml/site) on the clipped dorsal skin. Three hours later, compounds or DSCG (50 mg/kg.) were administered intraperitoneally while control group was given an equal quantity of distilled water.

One hour after the drug administration, 0.5 ml solution containing 1 % egg albumin along with 0.5 % Evan's blue dye solution was injected into tail vein of rats of all groups. Forty five minutes after antigen (egg-albumin) challenge, the rats were sacrificed and skins were removed and blue coloredarea due dye leakage on the each testing site of inner aspect skin was measured with the help of scale and caliper. The anti PCA protection offered by different drugs was calculated by following formula:

Percentage protection = 100 - Blue coloured area in treated group/ Blue coloured area in control group) ×100

Histamine Release Measurement Method [42]

The anti-allergic effect of the test samples on the induced histamine release from the human basophilic cell line, KU812 was evaluated. Briefly, KU812 cells (1.5x106 cells) were washed and resuspended in a Tyrode buffer. 5.0 mM calcium ionophoreA23187 (Wako, Osaka, Japan) was incubated with the test compounds (1.5m g/ml) and then added to the cell suspension. The mixture was incubated at 37°C for 20 min, and the reaction was terminated by cooling at 4 °C for 15 min. The cell suspension was then centrifuged and the amount of histamine in the supernatant was measured by the HPLC peak area using Cosmosil 5C18-PAQ. The percentage histamine release was calculated as follows:

Histamine release (%) = (test-negative control) / (positive control-negative control) X 100.

The supernatant from the nonresponsive cells was used as the negative control, and the supernatant from the stimulated cells only with A23187 was the positive control. IC50, the concentration that inhibited histamine release from the KU812 cells by 50% relative to control, was interpolated from graphed dose response results (1.5, 30, 75, 105mg/ml). IC50 values were determined graphically.

Modified Method [43]

Twenty four albino rats (150-250 g) of either sex of Haffkine's strain were sensitized with Freund's adjuvant (Calbiochem U. S. A.) and bovine albumin (Sigma Chemical Co. U. S. A.) 0.05 ml each. Ten days later the animals were sacrificed and the lungs were removed and perfused. The changes in the rate of flow were recorded after challenging with bovine albumin 0.05 ml in the perfusion system. In one group of 6 rats, 0.5 ml/100 g of the test compounds were injected intraperitoneally daily for 3 days prior to sensitization of rats. In the second group of sensitized Rats test compounds were administered

directly into the perfusion system in a dose of 0.5 ml/100 g of lung tissues. In the third group of rats the test compounds were added into the perfusion system in normal rats. In the fourth group of rats, disodium chromoglycate was added into the perfusion system (5 mg/100 g of lung tissue). In all the groups of rats, changes in the rate of flow were measured and results were analyzed [43].

Biological Assay

Materials

Goat anti-mouse IgE (GAME) and biotinylated GAME (b- GAME), murine and human recombinant IL-4Anti-mouse, anti-human CD40 antibodies and female 8-12-week-old BALB/cByj mice were collected. Spleen cells were obtained after sacrificing the mice by cervical dislocation, removal of the spleens, dispersing cells in a tissue grinder, filtering through nytex, washing twice with phosphate buffered saline (PBS), and finally culturinginDMEM/10% fetal bovine serum (FBS) at 37 °C, 10% CO2 [44].

Ex vivo IgE Assay

The *ex vivo* IgE response assay involved *in vivo* antigen priming and measurement of secondary antibody responses *in vitro*. The basic protocol was thoroughly documented and optimized for a range of parameters including: antigen dose for priming and time span following priming; number of cells cultured *in vitro*, antigen concentrations for eliciting secondary IgE (and other Igs) response *in vitro*, FBS batch that will permit optimal IgE response *in vitro*, showing the importance of primed CD4-positive T cells and hapten-specific B cells and specificity of the ELISA assay for IgE. Female BALB/cByjmice were immunized intraperitoneally with 10 μg dinitrophenol-conjugated keyhole limpet haemocyanin (DNP6-KLH) adsorbed onto 4 mg alum and sacrificed after 14–20 days. Spleens were removed and homogenized in a tissue grinder, washed twice. Spleen cell cultures (3×106 lymphocytes per ml) were established in 96-well round- bottom plates in the presence or absence of DNP6-KLH (10 ng/ml). Test compounds were added to spleen cell cultures immediately prior to the addition of antigen. Cultures were prepared in quadruplicate and incubated for 7-12 days.

IgE Response in Human Cells

Freshly drawn human peripheral blood was diluted 1:1 with sterile PBS and layered over 10 ml of Histopaque. The tubes were centrifuged at 1800 rpm (400 × g) for 30 min at room temperature. The plasma layer (yellow) was aspirated and the white cells were collected by removing the interphase. That layer was transferred to a new 50 ml conical tube containing 20 ml of sterile PBS. White blood cells were counted, centrifuged at 1000 rpm (250 × g) for 10 min, and re-suspended in 15 ml of sterile PBS. Human PBL cultures (5 × 105 cells per ml) were established in quadruplicate in the presence of human IL-4 and anti- human CD40 Ab (Pharmingen), and incubated at 37 °C; 10% CO2 for 10-14 days before harvesting supernatants for the ELISA.

Ig ELISA

To detect DNP-specific antibodies in cultures of mouse spleen cells ELISA plates were prepared by coating with specific antigen (DNP-OVA) overnight. After washing and blocking the plates with 200 μl bovine serum albumin (BSA) in PBS, an aliquot (1:4 dilution in PBS with 1% BSA 0.1% azide 0.5% Tween 20) of each culture supernatant were transferred directly to the ELISA plates and incubated overnight in a humidified box at 4 °C. IgE was quantified following successive 90 min incubations with biotinylated GAME (prepared in-house), alkaline phosphatase-streptavidin (Zymed), and 100 μl of phenolphthalein monophosphate (PPMP, DCHA salt, 40mg/ml). Absorption was measured at 540nm. The level of detection for IgE is about 200-400 pg/ml and cross- reactivity was less than 0.001% with any other Ig isotype in the ELISA for IgE.

Cytokine Assays

Drug activity was tested in two distinct assay systems utilizing spleen cells from female BALB/c mice. For the *in vitro* assay, T cells were isolated using Pan-T (CD3ε) magnetic bead kit (MiltenyiBiotec) and cultured for 48 h in the presence of ConA (5 μg/ml) in the presence and absence of drug. Culture supernatants were tested for IL-4 and IL-5 content using fluorescent beads from a cytokine detection kit (Upstate) and analyzed by a Luminex 100 flow analyzer. IL-4 and IL-5 to be quantify by comparison to standard

curves. For *ex vivo* cytokine response assay spleens were removed from mice that had been sensitized to DNP6-KLH. Two weeks following sensitization, mice were sacrificed and spleen cell cultures were established in the presence of DNP6-KLH. After 4 days, ConA (5 mg/ml) was added to cultures and the cells were incubated for an additional 4 h. Cytokine levels in the culture supernatants were quantified by ELISA using reagents and protocols were supplied by Pharmingen. Control responses were determined from cultures that contained no drug.

CONCLUSION

Thorough various studies of screening methods for anti - asthma activities might give some valuable information towards further research so, that it gives novel therapeutic development of effective and potent anti – asthmatic agents.

LIST OF ABBREVIATIONS

ELISA = Ezyme-linked Immuno-sorbent Assay

WHO = World Health Organization

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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