Antiallergic/antiasthmatic effect of novel antiallergic hexapeptide-95/220 in various experimental models

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The effects of newly synthesized antiallergic hexapeptide 95/220 was investigated on various allergic and asthmatic test models. This newly developed peptide was found to be more potent than clinically used drug disodium cromoglycate (DSCG). Hexapeptide 95/220 inhibited immediate hypersensitivity reactions such as passive cutaneous anaphylaxis (PCA) and mast cell degranulation in rats, antigen-induced bronchoconstriction in actively sensitized guinea pigs in dose dependent manner like DSCG. Antigen-induced contraction of guinea pig ileum was also markedly inhibited by this newly developed hexapeptide in the same fashion as ketotifen and DSCG did but at comparatively lower dose. Egg albumin-induced histamine release was also blocked by this hexapeptide from chopped lung tissues of sensitized guinea pigs. These results suggest that hexapeptide' 95/220 has potent inhibitory effect on immediate hypersensitivity reactions thereby inhibiting mediator release from mast cell. Moreover, this newly synthesized peptide is orally active and effective at lower doses as compared to standard drugs.

The number of deaths from asthma is rising and these alarming increases are occurring despite a marked increase in prescribed asthma therapy. The tissue injury within the airway wall in asthma is due to the influence of chemical mediators arising from infiltrating inflammatory and resident cell types. For many years mast cells have been known to play a central role in the pathogenesis of asthma. The traditional understanding has been that these cells are activated as a result of the interaction of allergen with IgE coated mast cells. This in turn releases a series of chemical mediators including histamine, leukotrienes (LTs), platelet activating factor (PAF), thromboxane A2, and prostaglandin (PG) D2 have significant roles in airway inflammation responses such as airway eosinophilia, late asthmatic response and airway hyperresponsiveness as well as in immediate hypersensitivity reaction such as bronchial constriction and airway plasma extravasation.

Novel therapeutic approaches based on new mechanisms of action are being developed with more emphasis on the prevention and cure of disease. In this direction, various peptides, immunosuppressants, neuropeptide antagonists IL-1 inhibitors, blockers inhibiting binding of IgE to mast cells have been recently reported. Inhibitors of IgE and its high affinity receptor FcεRI, appears to be an interesting approach for blocking allergic response.

From therapeutic point of view, IgE-Fc fragment would be able to prevent activation of the mast cells by interfering with the binding of the receptor and in terms about the early events that follow triggering of receptors. Although various pharmacologically active agents have been investigated as antiallergic-antiasthmatic agents but drug of choice to date remains limited up to symptomatic relief. Newly synthesized hexapeptide (Ala-Gly-Gly-Asp-Gly-Lys) have been found to have potent antiallergic/antiasthmatic effects in rats and guinea pigs among the series of other oligopeptides screened for developing as antiallergic and antiasthmatic drug.

Materials and Methods

Animals—Male Sprague-Dawley (S.D.) rats (120-150 g) and male English albino guinea pigs (325-375 g) were provided from animal house of Central Drug Research Institute, Lucknow.

Chemicals—Egg albumin (Grade V), compound 48/80, o-phthalaldehyde, toluidine blue and RPMI-1640 were purchased from Sigma Chemical Co., USA.

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Among drugs, disodium cromoglycate was manufactured by Rallis India Ltd., Mumbai and Ketotifen and cetirizine was obtained from Micro Labs Ltd., Bangalore. Novel antiallergic oligopeptide which is investigated in the present study was synthesized in Medicinal Chemistry Division, Central Drug Research Institute, Lucknow. These drugs were dissolved in 0.9% saline for oral administration.

The present study was conducted with peptide 95/220 which was synthesized by solid phase method and purified to more than 98% column followed by diverse phase HPLC.

PCA in rats—Egg albumin (EA) was used as antigen. Rat antiserum was prepared by sensitizing them on 1st, 3rd and 5th day. On 10th day of sensitization, serum was separated from the blood taken from the orbital plexus of rats and 1:10 dilution of serum containing IgE type of antibodies was used for sensitization.

Male SD rats were sensitized intradermally on their shaved backs with 0.1 ml of antiserum containing IgE. After 48 hrs. of sensitization, test agent (0.5 to 7.5 mg/kg) and standard drug (50 mg/kg) were given orally and intraperitoneal routes respectively. One hour later, 1% egg albumin along with 0.5% Evan's blue (0.25 ml each) were administered intravenously. After half to one hour, the inhibitory effects of test drugs were measured as the percent reduction in dye leakage area and compared with that of vehicle treated animals.

Mast cell stabilising activity—Compound 48/80, a condensation product of p-methoxy-N-methylphenylamine was used as potent histamine releaser in the non-immunological type of mast cell degranulation.

In the present investigation, normal saline (10 ml) was injected into the peritoneal cavity of SD rats. After gentle massage, the peritoneal fluid was collected and transferred into the siliconised - test tubes containing 10 ml of RMP I-1640 (pH 7.2-7.4).

The test peptides (0.1 to 0.5 mg/kg p.o. × 5 days) and standard (10 mg/kg, ip × 5 days) drugs were given to rats according to experimental protocol, prior to collection of mast cells. Mast cells were washed thrice by centrifugation at low speed (400-500 rpm) discarding the supernatant and taking the pellet of mast cells in the medium.

In non-immunological type of mast cell degranulation, mast cells from normal and treated groups were recovered, purified and were incubated with compound 48/80 (1 µg/ml) at 37°C for 10 min.

After incubation, mast cells were stained with toluidine blue (0.1%) and per cent protection against degranulation was counted under a high power microscope (x450)\(^ {14}\).

Active anaphylaxis (Schultz-Dale study)—Male guinea pigs were sensitized with egg albumin (10 mg/kg, ip) on days 1 and 3. On day 14, animals were sacrificed and ileum was surgically removed under aseptic condition and ileal strips were intensively washed with Tyrode's solution and put on a Schultz- Dale apparatus and incubated there at 37°C in Tyrode's solution into which air was bubbled. Motility of the segment of the ileum was recorded by Grass Model 7 Polygraph. As soon as the spontaneous motility (amplitude, frequency and tone of contractions) reached a constant level, tissue viability was tested by histamine hydrochloride (0.25 µg/ml). Antigen (10 µg/ml) was added to the bath (capacity 20 ml) and responses were recorded for 90 seconds. Various concentrations of test drugs were added to the organ bath 10 min before the addition of antigen (EA). The inhibitory effects of test drugs were expressed as the percent reduction in ileal contraction compared with that measured when the ileum was treated with the EA only (control)\(^ {15,16}\).

Allergic asthma and histamine aerosol test—Male guinea pigs were sensitized with antigen (EA, 50 mg/ml, im) on 1st, 3rd and 5th day. After 4 weeks of sensitization test peptide (5 mg/kg, po) was administered 1 hr prior to challenge. Then the sensitized and normal animals were kept in the aerosol chamber and an atomised mist of antigen (EA, 5%) and histamine (2%) were separately sprayed by nebulizer under pressure at 280 mm of Hg with the help of compressor. Guinea pigs were passively sensitized for EA and normal guinea pigs for histamine are employed in these tests. Animals exposed to broncho-constrictor aerosol behave in a characteristic manner and show progressive signs of difficulty in breathing and anoxic convulsions. As soon as preconvulsive breathing commences, the animals are placed in fresh air and the time is noted. Protection afforded by the drug is calculated as \[(1-T_1/T_2)\times100\]  where \(T_1\) is a mean of control preconvulsion time and \(T_2\) is post-treatment convulsion time\(^ {17}\).

Release of histamine from lung tissues—Egg albumin (2 mg/ml) and Freund's complete adjuvant were thoroughly mixed at the ratio of 1:1 by volume, and the resultant emulsion was used for sensitization. Male guinea pigs were actively sensitized by
intradermal injection of 1 ml of the emulsion on the shaved dorsal surfaces. After three weeks of sensitization, the animals were exsanguinated, and the lungs were perfused with 20 ml of Ca²⁺ free Tyrode's solution and then removed. The lungs were then chopped into fragments. The chopped lung tissues (200 mg wet weight) were placed in tubes with 2 ml of ice-cold Ca²⁺ free Tyrode's solution and kept on ice. The reaction tubes containing the lung tissues were supplemented with CaCl₂ (1.8 mM) and incubated for 10 min at 37°C. After that the lung tissues were incubated with test drugs (1-500 µM/ml) or vehicle for 60 min. and then again with EA (2 mg/ml). After 15 min, the reaction was stopped by filtration of the medium through nylon mesh (100 µm). Histamine in the medium was determined fluorometrically¹⁸.

Results

Protection to passive cutaneous anaphylaxis—Peptide 95/220 exhibited dose-dependent inhibition of passive cutaneous anaphylaxis in rats when administered by oral route thereby showing antiallergic activity and its activity was compared with standard drug DSCG.

DSCG exhibited 78% anti-PCA activity by intraperitoneal route at 50 mg/kg whereas peptide 95/220 exhibited 45 to 95% anti-PCA activity in dose-dependent manner by oral routes and ED₅₀ value was 1.0 mg/kg (Fig.1).

Mast cell stabilization—Peptide 95/220 also exhibited mast cell stabilising activity when peritoneal mast cells were stimulated to degranulate by compound 48/80, a potent histamine releaser. Mast cell stabilizing activity of test peptide was also assessed by comparing with DSCG, a potent known mast cell stabilizer. Peptide inhibited the degranulation in dose-dependent manner and maximal activity (83%) was shown at 7.5 mg/kg in divided doses of 1.5 mg/kg for 5 days (Fig. 2). DSCG at 50 mg/kg ip (10 mg/kg for 5 days) showed 68% protection of mast cells.

Effect on active anaphylaxis (Schultz-Dale test) in guinea pigs—Peptide 95/220 and the reference drugs inhibited antigen-induced contraction of isolated guinea pig ileum in concentration dependent manner.

Peptide 95/220 showed maximum protection to contraction of ileal strip at 5 µg/ml (72%) and blocked the contraction (20-72%) of ileal tissue in dose-dependent manner (1-5 µg/ml).

Protection to antigen-induced contraction of guinea pig ileum produced by peptides was compared with standard drug DSCG, ketotifen and cetirizine which also blocked the contraction in dose-dependent manner (Figs 3-6).

DSCG protected antigen-induced contraction of sensitized guinea pig ileum and maximum protection was exhibited at 6 µg/ml which blocked contraction up to 72% whereas ketotifen and cetirizine exhibited 93% and 95% protection at very low doses, i.e. 2.5 and 3 µg/ml respectively.

Effect on bronchodilator test—Bronchodilatory activity of peptide 95/220 has been evaluated in both sensitized and normal guinea pigs in antigen and histamine-induced aerosol test at higher dose (5.0 mg/kg, po) and its activity have been compared with standard drug DSCG ( administered intraperitoneally at 50 mg/kg) and cetirizine (1.0 mg/kg, po). Antihistaminic activity have also been evaluated for peptides and DSCG and not for cetirizine because it is a known antihistamine drug (Table 1).

Effect on antigen induced histamine—Peptide 95/220 inhibited antigen-induced release of histamine from chopped lung tissues of actively sensitized guinea pigs in a concentration dependent manner, and maximum inhibition was observed at 10 µM. Ketotifen showed least inhibition as compared to DSCG and peptide 95/220. The inhibitory effect of DSCG was observed from 100-500 µM whereas...
peptide 95/220 exhibited equal potency at 10 μM, (Fig. 7).

**Discussion**

In the present experimental studies, the effects of newly developed antiallergic peptide 95/220 on immediate hypersensitivity reaction were investigated using various experimental models and airway responses. Degranulation of mast cells when exposed to antigen, has been taken as the criterion of positive anaphylaxis. It was found that oral administration of 95/220 was effective in inhibiting immediate hypersensitivity at very low dose as compared to standard drug DSCG which is effective by intraperitoneal route at much higher dose.

Peptide 95/220 showed substantially higher potency in inhibiting the PCA reaction in rats than did DSCG. The inhibitory effect of 95/220 on antigen-
induced bronchoconstriction in guinea pigs was more potent than DSCG at much higher dose and less or equally potent to cetirizine.

Further, Peptide 95/220 exhibited protection to mast cell degranulation when induced by a potent histamine releaser compound 48/80, thereby acting as mast cell stabilizer. However, till Naguchi's peptide it was controversial whether the pentapeptide synthesized by Hamburger is able to compete with IgE in binding to mast cells and basophils to impair antigen-induced histamine release from mast cells or inhibit PCA reaction.

Nio et al. carried out the synthesis of some peptide fragments and these peptides were assayed for their capacity to inhibit PCA in vitro. The results suggested that an octapeptide corresponding to 345-352 in the human IgE molecule may be an IgE binding site. It exhibited significant inhibition of PCA, probably by occupying the Fc epsilon receptor sites on the cells.

Anti-PCA activity of peptide 95/220 was investigated by administering it through intraperitoneal route and its activity was compared with DSCG. Peptide 95/220 exhibited better anti-PCA activity by ip route as compared to oral route. The main advantage of peptide over clinically used cromoglycate is that it is at least 50 times more potent dose per dose than DSCG.

Further it is postulated that anti-anaphylactic effect of DSCG is due to its ability to inhibit the release of mediators from mast cells. This inhibition of mast cell degranulation and mediator release has been demonstrated in animal studies in response to both immunological and non-immunological stimuli and in humans following allergen stimulation.

In active anaphylaxis (Schultz-Dale study) peptide 95/220 inhibited antigen-induced contraction of ileal tissue and its activity was compared with standard drugs DSCG, ketotifen and cetirizine. Peptide 95/220 and DSCG were found to be equipotent. They have inhibited egg albumin-induced contraction at same dose whereas ketotifen and cetirizine have blocked the contraction at lower doses as compared to DSCG and peptide 95/220. These results also indicate that peptide also possess mast cell stabilizing activity as does DSCG.

In general, experiments for evaluating bronchodilatory activity of peptide was also compared with DSCG and cetirizine possessed bronchodilatory activity approximately in same range by oral route unlike DSCG which was given by intraperitoneal route and much higher dose range. Peptide as well as DSCG do not have anti-histaminic activity as these have shown only 17% inhibition to bronchoconstriction when normal guinea pigs were exposed to histamine spray through nebuliser.

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<th>Table 1 — Bronchodilator study - Aerosol test</th>
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*Cetirizine is known antihistaminic so its antihistaminic activity was not determined in our laboratory. (n = 5 in each group)*

![Fig. 7 — Effect of peptide 95/220 (●), DSCG (▲) and Ketotifen (■) on antigen released histamine from chopped lung tissues of sensitized guinea pigs.](image-url)
Whereas known antihistaminic drug cetirizine also inhibited antigen-induced bronchoconstriction in sensitized guinea pigs. These results indicate that peptide 95/220 like DSCG have ability to stabilise mast cell membrane by competitively binding with mast cell membrane receptors but do not have ability to antagonise the effect of released mediators. Unlike clinically used drug DSCG, this peptide is orally active at much lower dose.

Peptide 95/220 markedly inhibited immediate hypersensitivity reactions such as antigen-induced cutaneous reaction, mast cell degranulation induced by non-immunological stimuli (Compound 48/80), Schultz-Dale phenomenon and bronchoconstriction when given orally. These effects of 95/220 may be beneficial for the treatment of allergic inflammatory diseases such as bronchial asthma, allergic rhinitis and allergic dermatitis in human. Peptide 95/220 and DSCG inhibited histamine release from the chopped lung tissues of actively sensitized guinea pigs which showed their antiallergic activity. Many Histamine antagonist compounds such as epinastin23 and ketotifen24 have been found to suppress antigen or calcium ionophore induced release of chemical mediators. It has been suggested that the inhibitory effects of the antiallergics on the chemical mediator release could be ascribed to the inhibition of cellular Ca²⁺ uptake25,26 and the enhancement of the cellular cyclic AMP level27,28. It is necessary to examine the effects of peptide 95/220 on the second messengers to clarify the mechanism of chemical mediator release inhibition.

Thus, our studies provide the first experimental evidence for the presence of antiallergic activity in small peptides by oral route and open a new avenue for future exploration. Therefore, small peptides comprised of 5-10 amino acid residues which can interact with airways immune response may be of significant interest as lead molecules for realising the long awaited goal of preventive therapy of asthma. With this in mind detailed studies with clinically established immunomodulatory peptides may lead to better therapeutic modalities against allergic diseases.

References
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