

Studies on the anti-allergic action of N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5')

R. HAMEL, A. W. FORD-HUTCHINSON and H. PIECHUTA

Department of Pharmacology, Merck Frosst Canada Inc., Pointe Claire-Dorval, Québec, Canada

The effects of the anti-allergic compound N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5' or Tranilast) have been investigated upon antigen-induced contractions of respiratory smooth muscle *in vitro* and bronchoconstriction *in vivo*. N-5' (3.1×10^{-5} M) *in vitro* had no significant effect upon antigen-induced contractions of the guinea pig trachea. However, when tested at concentrations of 9.2 and 3.1×10^{-5} M, but not 0.9×10^{-5} , N-5' significantly inhibited antigen-induced contractions of the human parenchyma. *In vivo* N-5' (5 and 10 mg/kg p.o.) failed to inhibit antigen-induced dyspnea in hyperreactive rats. N-5' (20 mg/kg p.o.) also failed to inhibit the immediate bronchoconstriction following ascaris challenge of conscious squirrel monkeys but significantly attenuated the reduction in dynamic compliance during the pulmonary late phase response observed between 4 and 10 h after antigen challenge. It is suggested that N-5' does not act as a "cromoglycate-like" compound but may have novel mechanisms of action in human bronchial asthma.

Key words: anti-allergic drugs; N-5'; Tranilast; antigen-induced bronchoconstriction.

Accepted for publication 4 December 1986

Pharmacological studies on N-(3'-4'-dimethoxycinnamoyl) anthranilic acid (N-5' or Tranilast) have shown that this compound has anti-allergic properties (1, 6, 9). Clinical studies have suggested that N-5', an orally active drug, has clinical activity in the treatment of allergic bronchial asthma (13, 14). It has been suggested that N-5' has similar properties to disodium cromoglycate and that the mechanism of action involves prevention of mediator release through "stabilization" of the membranes of mast cells and basophils (1, 9, 10). However, two pieces of evidence argue against this interpretation. First, the doses of N-5' required in animal models to exert anti-allergic activity are well in excess of those used in man (1, 6, 9). Secondly, the concept that disodium cromoglycate works through a mechanism involving "stabilization" of mast cells has been called into question (16). In the present study we have investigated further the mechanism of action of N-5'.

METHODS

In vitro studies

Sensitization of guinea pigs. Male guinea pigs (300-400 g) were sensitized by injecting intraperitoneally 0.5 ml of a solution containing 10 µg of egg albumin and 0.1 g aluminium hydroxide dissolved in saline. Two weeks were permitted for sensitization to occur and the animals were used 14 to 30 days post-sensitization.

Sensitization of human tissue. Macroscopically normal parts were selected from three separate human lung parenchyma, excised because of carcinoma, and immediately after removal placed in cold saline. The parenchymal strips were prepared by cutting lung strips (peripheral portions of the lung sample) which were approximately 4-5 mm wide and 2-3 cm long. The tissues were stored overnight at 4°C in

sealed bottles containing Krebs' buffer previously gassed with 95% oxygen and 5% carbon dioxide together with serum (1/10 final concentration) of patients allergic to ragweed.

Tissue preparation. Sensitized guinea pigs were stunned and exsanguinated. The trachea were removed, freed of adhering tissue and divided longitudinally by cutting through the cartilaginous tissue directly opposite the muscle insertion. Each opened trachea was then transected between every second cartilage. Four of the cut sections were tied together, end to end, in a series with # 0.7 silk thread ensuring that the tracheal muscles were all in the same vertical plane. Each chain consisted of tissue selected from three different animals. The guinea pig trachea and human parenchyma were suspended under 1 g of tension (by silk ties at each end) in a 20 ml organ bath containing 10 ml of modified Krebs' buffer at $37 \pm 0.5^\circ\text{C}$, gassed with 95% oxygen and 5% carbon dioxide. The composition of the modified Krebs' bicarbonate buffer in millimolarity (mM) was: KCl 4.72; CaCl_2 2.5; MgSO_4 0.7 H_2O 0.5; KH_2PO_4 1.2; NaCl 120; dextrose 11; NaHCO_3 25. The pH of the bathing solution was 7.35 ± 0.05 . Mepyramine (0.55 $\mu\text{g/ml}$) and indomethacin (2.67 $\mu\text{g/ml}$) were added to the buffer to inhibit the contribution of histamine or prostaglandins to the contraction. To record responses, one end of the tracheal chain was attached to a Gould-Statham UC-2 force displacement transducer which was connected to a Beckman Type R-dynograph. The preparations were allowed to equilibrate for 1 h, during which time the tissues were automatically washed (10 ml volume displacement) every 6 min. After the equilibration period, tracheal chains and parenchymal strips were primed respectively with methacholine (3 $\mu\text{g/ml}$) and U-44069 (5 $\mu\text{g/ml}$), washed and allowed to recover to baseline. Two tracheal chains were used as a control. These were challenged with a concentration of egg albumin sufficient to induce an average contraction of 65–85% of the methacholine response. N-5' was added to the tissues 15 min prior to challenging the fresh

chains with the same dose of egg albumin as used in the controls. N-5' was dissolved in dimethyl sulfoxide and the volume added to the bath was 10 μl . The response of the challenged tissue was expressed as a percentage of the methacholine maximum and the % inhibition was then calculated. Parenchymal strips were treated with N-5' or vehicle and challenged 15 min later with 100 μl of ragweed extract. The activity of the compound was reported as percentage of tissues that responded to antigen rather than as percentage of maximal contraction. In the control group ($n = 9$), contractions of parenchymal strips induced by ragweed varied between 0 and 83% of U-44069 maximum contraction.

In vivo studies

Antigen-induced dyspnea in hyperreactive rats. Inbred rats (15–18 generations), hyperreactive to aerosolized antigen, were obtained from this laboratory (2, 8). Rats were sensitized to produce IgE antibodies, challenged with an aerosol of antigen and the respiratory responses recorded as the duration of dyspnea as previously described (12, 15).

Rats were pretreated with N-5' as a suspension in methocel (0.4% w/v) and Tween 80 (0.5% w/v) or the drug vehicle 1 h prior to exposure to antigen.

Ascaris-induced bronchoconstriction in conscious squirrel monkeys. The measurement of dynamic compliance (C_{dyn}) and pulmonary resistance (R_{L}) in conscious squirrel monkeys has previously been described (7, 12). Four squirrel monkeys were used in the present study and these monkeys were challenged with an aerosol of the ascaris antigen (50,000 or 100,000 pulmonary nitrogen units/ml) on a regular basis at intervals no shorter than 2 weeks. The aerosol was generated using an ultrasonic nebulizer, model 25C, (The DeVilbiss Co., Somerset, PA.) and delivered to the animal through the face mask for a period of 6 min. Pulmonary mechanical changes were recorded for up to 13 h during which time stable control base lines were obtained, the animals

treated with drug, challenged with antigen and pulmonary mechanics recorded for 10 h after challenge. All four monkeys showed reproducible responses to repeated ascaris antigen challenges over a period of 10 months. For control purposes the mean responses to 3–4 consecutive separate challenges in the absence of drug were used. Monkeys were treated with N-5' (20 mg/kg) as a suspension in 0.4% (w/v) methocel and 0.5% (w/v) Tween 80 p.o. 1 h prior to exposure to ascaris antigen. Mean values for R_L and C_{dyn} were calculated at 5-min intervals prior to and for 1 h after antigen challenge. Results after challenge with ascaris were expressed as a percentage change relative to the baseline values. The time at which the late phase response was maximal varied between experiments. To group the data together, the peak of the late response on each individual experiment was designated as time 0. Results expressed as percentage changes relative to the baseline values were then calculated at 15 min intervals before and after the peak response. These calculations were carried out separately for R_L and C_{dyn} because in 50% of the experiments the peak increases in R_L occurred prior to the peak decreases in C_{dyn} . For statistical analysis for each animal the values of R_L and C_{dyn} from 120 min before the peak of the late phase to 90 min after the peak were meaned. The mean values were then tested for normality (Wilk-Shapiro test) and analysed by the paired t-test.

MATERIALS

Egg albumin, grade V, salt free, lyophilized (Sigma), aluminium hydroxide (Reheis Chemical Co.), *Ascaris suum* antigen (Greer Laboratories), ragweed extract (200,000 pulmonary nitrogen units/ml) (Omega Laboratories), methacholine chloride (Sigma Chemical), U-44069, a stable prostaglandin endoperoxide analogue (15(S)-hydroxy-9 α ,11 α -(epoxy methanol)-prosta- 5-cis, 13 trans, dienoic acid) (Upjohn), indomethacin, and mepyramine (Merck & Co. Inc.), N-5' (Kissei).

RESULTS

Inhibition of antigen-induced contractions of the guinea pig tracheal chain

Antigen challenge of sensitized (IgG antibodies) guinea pig tracheal chains in the presence of indomethacin and mepyramine induces contractions which are thought to be largely due to the release of 5-lipoxygenase derived products of arachidonic acid metabolism (3). Following exposure to ovalbumin (0.01 μ g/ml) a contraction equivalent to 65% of methacholine maximum was observed. N-5' at a dose of 10 μ g/ml (3.1×10^{-5} M) in the presence of mepyramine (0.55 μ g/ml) produced at $12 \pm 5\%$ (mean $\pm$ SEM, $n = 5$) inhibition of ovalbumin-induced contractions of guinea pig tracheal chains.

Inhibition of antigen-induced contractions of the human parenchymal strip

Human parenchymal strips, passively sensitized overnight at 4°C with serum of ragweed-allergic patients produced contractions following challenge with ragweed antigen. In the control group, 89% (8/9) of the tissues responded to ragweed challenge, whereas following pretreatment of the tissues with 30.0 (9.2×10^{-5} M) ($n = 4$) and 10.0 (3.1×10^{-5} M) ($n = 3$) μ g/ml of N-5' no contraction was observed. At a lower dose, N-5' (3.0 μ g/ml; 0.9×10^{-5} M) failed to prevent the induced contraction ($n = 2$).

Inhibition of antigen-induced dyspnea in hyperreactive rats

Rats hyperresponsive to spasmogenic mediators (2) were sensitized with ovalbumin to produce IgE antibodies. Following exposure to an aerosol of antigen, these rats showed reproducible episodes of dyspnea which were quantified by measuring the duration of dyspnea in minutes. The duration of dyspnea in the control group was 19.4 ± 3.2 min (mean $\pm$ SEM, $n = 5$). Pretreatment of the animals with either 5 or 10 mg/kg N-5' (p.o.) produced no significant reduction in the duration of the dyspnea (5 mg/kg, 18.3 ± 5.7 min and 10 mg/kg 12.3 ± 8.5

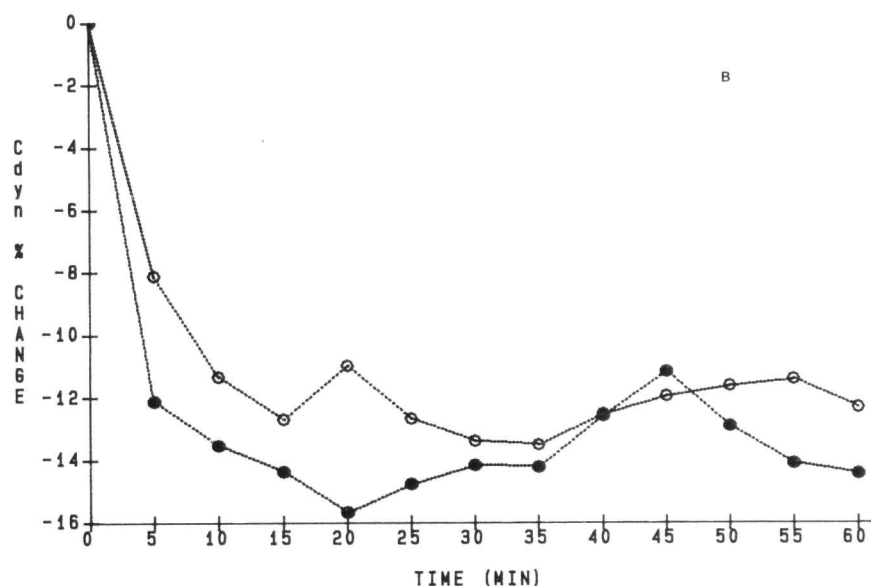
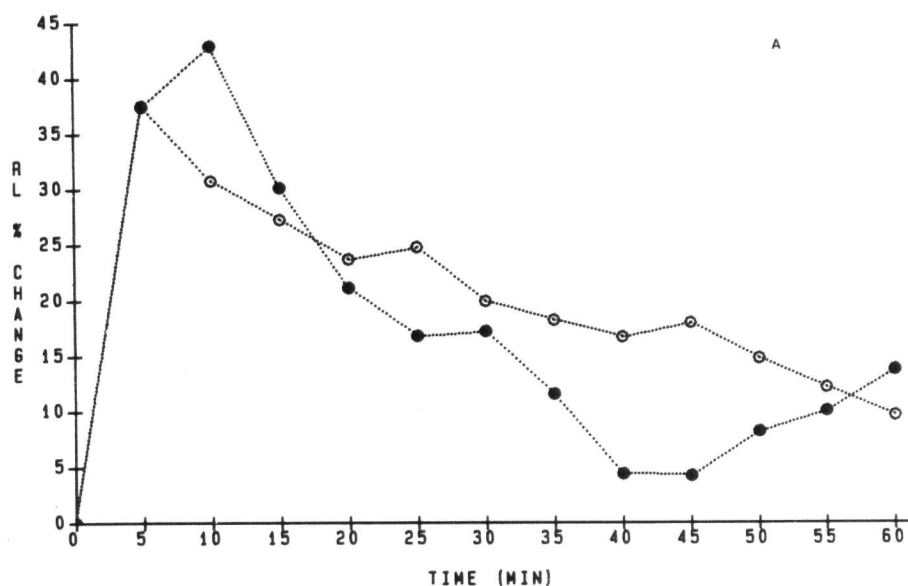


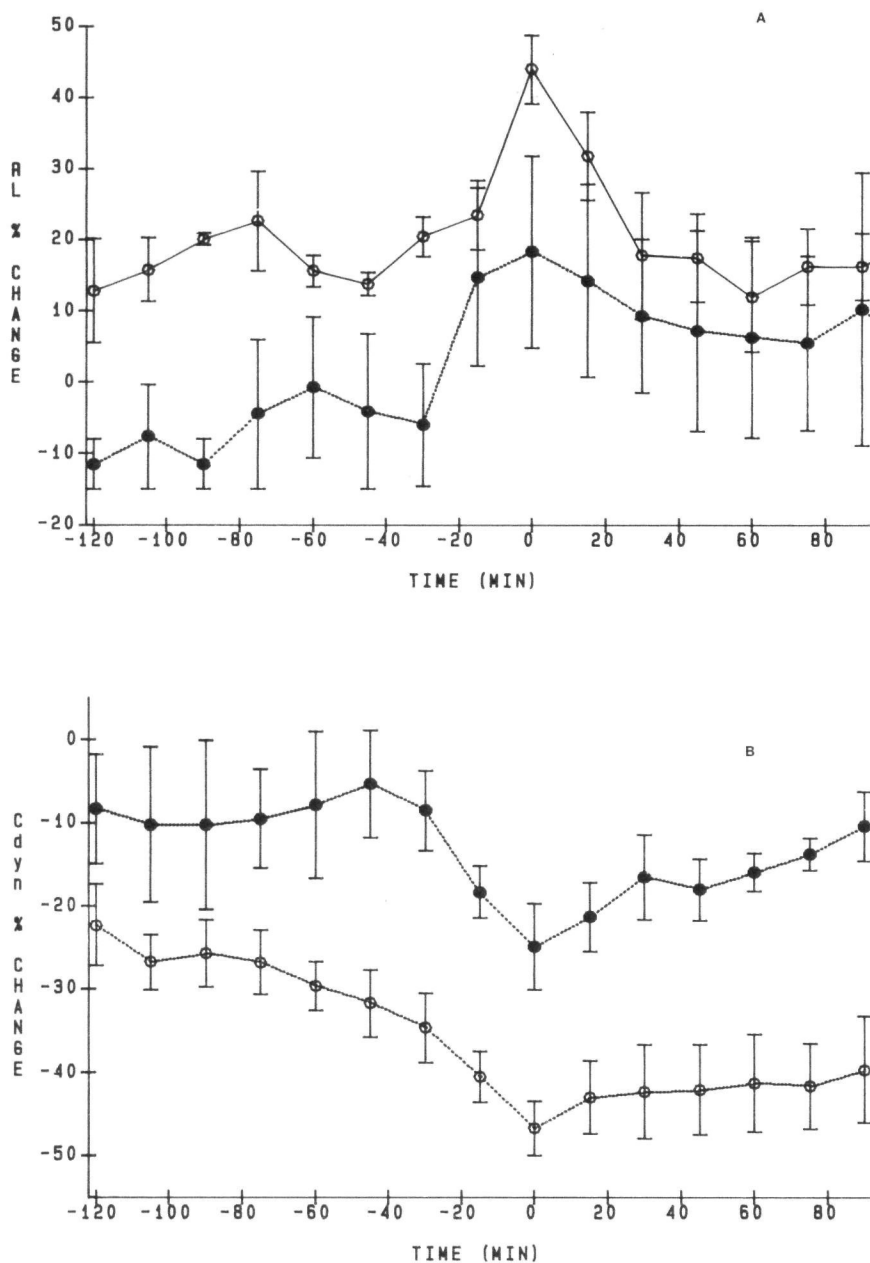
Fig. 1A and 1B. The effects of N-5' on ascaris-induced immediate bronchoconstriction in conscious squirrel monkeys (Fig. 1A, increases in R_L ; Fig. 1B, decreases in C_{dyn}). Treatments were as follows: control (○), N-5' (●) (20 mg/kg, p.o. - 1 h). Results are shown as means from 4 separate monkeys. Control data on each monkey represents the mean of 3-4 separate experiments.

min, results expressed as means $\pm$ SEM, $n = 5$) induced by the aerosol of antigen.

Inhibition of ascaris-induced bronchoconstriction in conscious squirrel monkeys

Exposure of conscious squirrel monkeys with known sensitivity to *Ascaris suum*, to an aerosol of ascaris resulted in immediate bronchoconstriction followed by a late pulmonary response 4 to 10 h later involving both the upper and lower airways (7). Following repeated exposure, these responses were reproducible over a 10-month period. Figs. 1A and 1B show the immediate pulmonary responses following exposure, which was characterized by prolonged increases

in R_L and decreases in C_{dyn} . Control data from 3-4 separate challenges was meaned for each monkey and then mean data from four separate monkeys was meaned to produce the results shown in Figs. 1A and 1B. Pretreatment of the animals with N-5' (20 mg/kg p.o.) had no effect on the baseline (data not shown) and produced no inhibition of the immediate pulmonary response induced by ascaris (Figs. 1A and 1B). After partial or total recovery a second bronchoconstriction commenced between 2 and 6 h (7) and the peak of the late response is illustrated on Figs. 2A and 2B. In four monkeys, pretreatment with N-5' prior to antigen challenge produced significant inhibition of these changes in C_{dyn} ($P < 0.02$). The inhibi-



Figs. 2A and 2B. The effects of N-5' on ascaris-induced late bronchoconstriction in conscious squirrel monkeys (Fig. 2A, increases in R_L; Fig. 2B, decreases in C_{dyn}. Time 0 min represents the peak of the late response for each individual experiment (between 4 and 10 h after antigen challenge). Treatments were as follows: control (○), N-5' (●) 20 mg/kg, p.o. administered 1 h prior to antigen challenge). Results are shown as means ± SEM from 4 separate monkeys. Control data on each monkey represents the mean of 3–4 separate experiments.

tion of the changes in R_L did not reach statistical significance (*P* < 0.09).

DISCUSSION

In the present study *in vitro* and *in vivo* models of human allergic asthma have been used to further profile the prophylactic activity of N-5'. The *in vitro* models utilized actively sensitized guinea pig trachea (IgG₁ antibodies) and passively sensitized human parenchymal strips (IgE antibodies). In both tissues the contraction induced following antigen challenge is attributable in part to the release of peptidolipid leukotrienes (3, 5). On the guinea pig trachea, the compound (10 µg/ml) produced weak inhibition

of the antigenic response whereas on human parenchyma N-5' (10 and 30 µg/ml) suppressed the antigen response. The results support previous *in vitro* reports demonstrating that doses higher than 3 × 10⁻⁵M are required to produce significant inhibition of antigen responses (10). The greater potency of N-5' on human parenchyma as compared to guinea pig trachea may be due to an enhanced ability to block IgE rather than IgG₁ response. Similar results have been reported for disodium cromoglycate (4). The first *in vivo* model utilized a strain of inbred rats which show non-specific bronchial hyperreactivity (2, 8), an important feature of human bronchial asthma. These rats develop reproducible, prolonged episodes of dyspnea

following antigen challenge (14). The second *in vivo* model used in the present study involved challenge of conscious squirrel monkeys with an aerosol of ascaris antigen. Recently we have demonstrated that certain squirrel monkeys challenged with antigen undergo a reproducible dual pulmonary response (7). In both *in vivo* models the antigen response appears to be IgE-mediated. Administration of N-5' (5–20 mg/kg p.o.) prior to antigen challenge produced no significant inhibition of the immediate pulmonary responses in either the inbred, hyperreactive rats or squirrel monkeys. However, in the primate model, N-5' produced significant inhibition of the changes in C_{dyn} and weak inhibition of changes in R_L occurring during the pulmonary late phase. In addition to blocking the peak late phase response, pretreatment with N-5' shortened the duration of the late phase.

The results in the present work are not inconsistent with previous data. Published *in vitro* data has demonstrated effects on antigen-induced release of mast cell-derived products only at high doses (9), explaining the relative lack of effect upon guinea pig tracheal chains. The effects on human parenchymal strips are of more interest as they occur at concentrations close to those found in human plasma following multiple dose administration ($\approx 20 \mu\text{g/ml}$). The therapeutic dose of N-5' used in man is between 6 and 12 mg/kg. At this dose no effect was seen upon immediate antigen responses in either the rat or the squirrel monkey in the present study. Previous studies in various models, such as the passive cutaneous anaphylaxis assay in the rat, have only demonstrated *in vivo* activity at doses ($> 50 \text{ mg/kg}$) (1, 6, 9) well in excess of those used either clinically (13, 14) or in the present study. It was, therefore, of particular interest to see an effect in the squirrel monkey model in the pulmonary late phase response. The pathogenesis of the pulmonary late phase is not clearly defined. However, leukotrienes B_4 , C_4 and D_4 , neutrophil chemotactic factor and thromboxane B_2 have all been implicated as mediators of the pulmonary late phase and this has been used as evidence for a role for mast-cell derived mediators in this response (11). It has been suggested that infiltration of inflammatory cells

is an important contributing factor to the late phase response (11), and one mechanism of action of N-5' may be through a blockade of the synthesis and/or release of non-spasmogenic mediators which in turn modulate the late phase response or modulate the cellular infiltration.

The data presented are not consistent with the concept that N-5' is an orally active "cromoglycate-like" compound with a mechanism involving "stabilization" of mast cells. The *in vitro* and *in vivo* results are not consistent with a potent effect on the primary release of mediators following antigen challenge; previous positive results having been obtained at doses well in excess of those used clinically (1, 6, 9). For example, disodium cromoglycate showed potent activity in the hyperreactive rat model ($ED_{50} 1 \text{ mg/kg i.v.}$) (15 and unpublished observations) whereas N-5' was inactive at 5 or 10 mg/kg p.o. In addition, the concept that disodium cromoglycate exerts therapeutic effects in man through "stabilization" of human mast cells has been questioned both because of basic studies carried out on disodium cromoglycate and because of the relative clinical inefficacy of more active oral "cromoglycate-like" compounds (16). Modulation of the late phase response may modulate the hyperreactive response and is a property shared with disodium cromoglycate and corticosteroids (11). Studies upon drugs such as N-5', which appear to selectively alter the late phase response, may provide new information as to the pathogenesis and implications of the late phase in human asthma.

REFERENCES

1. Azuma, H., Banno, K. & Yoshimura, T.: Pharmacological properties of N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5'), a new anti-atopic agent. *Br. J. Pharmacol.* 58, 483–488, 1976.
2. Brunet, G., Piechuta, H., Hamel, R., Holme, G. & Ford-Hutchinson, A. W.: Respiratory responses to leukotrienes and biogenic amines in normal and hyperreactive rats. *J. Immunol.* 131, 434–438, 1983.
3. Burka, J. F. & Paterson, N. A. H. Evidence for lipoxygenase pathway involvement in allergic tracheal contraction. *Prostaglandins* 19, 499–515, 1980.
4. Cox, J. S. G. Disodium cromoglycate. Mode of action and its possible relevance to the clinical use of the drug. *Br. J. Dis. Chest.* 65, 189–204, 1971.

5. Dahlén, S. E., Hansson, G., Hedqvist, P., Björck, T., Granström, E. & Dahlén, B.: Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C₄, D₄ and E₄. *Proc. Natl. Acad. Sci. USA* 80, 1712-1716, 1983.
 6. Fujimura, M., Koshino, T., Ishizaki, T., Minami, S., Saga, T. & Miyabo, S. Inhibitory effect of N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5') on SRS-A mediated bronchoconstriction in the guinea pig in vivo. *Allergy* 40, 98-102, 1985.
 7. Hamel, R., McFarlane, C. S. & Ford-Hutchinson, A. W.: Late pulmonary responses induced by ascaris allergen in conscious squirrel monkeys. *J. Appl. Physiol.* 61, 2081-2087, 1986.
 8. Holme, G. & Piechuta, H. The derivation of an inbred line of rats which develop asthma-like symptoms following challenge with aerosolized antigen. *Immunology* 42, 19-24, 1981.
 9. Koda, A., Nagai, H., Watanabe, S., Yanagihara, Y. & Sakamoto, K.: Inhibition of hypersensitivity reactions by a new drug, N(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5'). *J. Allergy Clin. Immunol.* 57, 396-407, 1976.
 10. Kubota, T., Ujiie, A., Naito, J., Nakazawa, M. & Koda, A. Studies on the mechanisms of the inhibitory effect of N-5' on histamine release from rat peritoneal exudate cells. *Jpn. J. Pharmacol.* 33, 837-843, 1983.
 11. Lemanske, R. F. & Kaliner, M.: Mast cell-dependent late phase reactions. *Clin. Immunol. Rev.* 1, 547-580, 1981-82.
 12. McFarlane, C. S., Piechuta, H., Hall, R. A. & Ford-Hutchinson, A. W.: Effects of a contractile prostaglandin antagonist (L-640,035) upon allergen-induced bronchoconstriction in hyperreactive rats and conscious squirrel monkeys. *Prostaglandins* 28, 173-182 (1984).
 13. N-5' internal medicine research group. Evaluation of the clinical efficacy of N-5' in bronchial asthma. A multi-clinic study by the double blind method. *Igaku no Ayumi (Strides in Medicine)* 108, 252-273, 1979.
 14. N-5' study group in children. A double blind controlled trial of N-(3',4'-dimethoxycinnamoyl) anthranilic acid on children with bronchial asthma. *Allergy* 34, 213-219, 1979.
 15. Piechuta, H., Smith, M. E., Share, N. N. & Holme, G.: The respiratory response of sensitized rats to challenge with antigen aerosols. *Immunology* 38, 385-392, 1979.
 16. Stokes, T. C. & Morley, J.: Prospects for an oral Intal. *Br. J. Dis. Chest* 75, 1-14, 1981.
- Address:
Dr. A. W. Ford-Hutchinson
Merck Frosst Canada Inc.
Department of Pharmacology
P. O. Box 1005
Pointe Claire-Dorval, Québec
Canada H9R 4P8

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.