Preclinical Evaluation of Antiallergic Agents

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Abstract □ This paper examines the advantages and disadvantages of many *in vivo* and *in vitro* experimental model systems employed in the preclinical testing of antiallergic drugs.

Keyphrases □ Antiallergic drugs—inhibition of mediator release, *in vivo* and *in vitro* models □ α-Adrenoceptor agonist—cyclic adenosine monophosphate levels, effects on mediator release, antiallergic drugs □ β-Adrenoceptor agonists—cyclic guanosine monophosphate levels, effects on mediator release, antiallergic drugs □ Asthma—review of current theories, clinical evaluation, symposium, antiallergic drugs □ Cromolyn sodium—prophylactic antiallergic drug

The primary purpose of this report is to provide insight into the drug discovery process by examining several commonly used animal models as well as to illustrate the type of data that may be obtained. It is not intended as a thorough review of all methods currently available for the detection of antiallergic activity of novel molecules. The merits and limitations of these various systems, at least as presently viewed, are addressed. Allergic asthma will serve as a focus for allergic disorders in general. It is hoped that agents efficacious in this disease will prove beneficial for other allergic conditions as well.

Most experimental animal models commonly are based on the "mediator concept" of allergic disorders discussed in the preceding paper (1). The rationale for this approach to pharmacotherapy lies almost totally with the known pharmacology of the prototype prophylactic antiallergic agent cromolyn sodium and its well-established clinical utility (2).

Before beginning a discussion as to the type of technology employed, it is pertinent to examine the question of why a search for antiallergic agents beyond cromolyn sodium should continue. Cromolyn sodium is not orally active and is, therefore, administered as a powdered aerosol by a clever but cumbersome technique. The drug is efficacious in only a portion of the asthmatic patients for reasons that are not presently understood (2). Consequently, a compound that is orally active or that can be administered by more conventional aerosol techniques and/or that can protect a broader group or preferably all asthmatics would be a significant advance in therapy.

IN VIVO MODELS

By far, the most widely used paradigm is the 48-hr rat passive cutaneous anaphylaxis test (3), which frequently serves as the primary laboratory experimental system (Fig. 1). Briefly, adult female rats are immunized on Day 0 with an appropriate dose of ovalbumin along with *Bordetella pertussis*, which serves as an adjuvant. Often, rats receive a subcutaneous injection of larva from the nematode *Nippostrongylus brasiliensis* several days after primary immunization to enhance the production of antiovalbumin reaginic antibody. The rats are killed and bled 10–14 days later, and serum containing the antibody is prepared.

Appropriate dilutions of the serum are subsequently injected into discrete areas of the dorsal surface of the skin of a recipient rat. Since certain subclasses of immunoglobulin G antibodies may also bind to cutaneous mast cells, a 48-hr period is interposed between sensitization and antigen challenge to allow these antibodies to dissipate. Immunoglobulin E-type antibodies directed against ovalbumin remain firmly fixed to the mast cells for much longer periods (4). Test compounds are administered to the animals immediately prior to challenge with ovalbumin, which is often mixed with Evans blue dye. The short time between administration of the test compound and antigen is essential to ensure optimal activity. Unaccountably, there is a well-established inverse relationship regarding the time interval between drug and antigen administration and efficacy (3).

Upon injection, the antigen interacts with the antibodies fixed to the mast cell surface, which leads to a series of intracellular biochemical events culminating in the release of the vasoactive amines, histamine and serotonin, from the cells. When the animal is killed 30 min later and the dorsal skin reflected, the pharmacological effect of these mediators is visualized as an area of dye infiltration (wheals), which can be quantitated. Antiallergic agents are expected to reduce mediator release, which is manifested as a reduction in wheal size as compared to controls. Those compounds that demonstrate efficacy often undergo secondary testing in the rodent. Wheal size reduction in rats may be brought about by interfering with the antigen-induced release of the mediators of anaphylaxis from mast cells or by antagonizing the ability of histamine and/or serotonin to increase vascular permeability. To discriminate between these types of activity, the ability of the test compound to block wheals produced by the direct intracutaneous injection of histamine and/or serotonin is evaluated. Compounds that antagonize the vascular response to histamine and/or serotonin are often discarded as having a potentially unsuitable mechanism of action. One shortcoming of this approach is the possibility that molecules that possess antiallergic as well as antagonist types of activity may be overlooked.

The principal advantages of the rat passive cutaneous anaphylaxis model are that it is simple, rapid, economical, and semiquantitative. Furthermore, the potential oral efficacy of compounds may be assessed at an early stage in the pharmacological testing sequence. Major disadvantages are that it provides only circumstantial evidence as to the mechanism of action of the compounds and that efficacy is established in cutaneous tissue rather than in the designated therapeutic site, pulmonary and tracheobronchial tissue.

Various *in vivo* models of "allergic asthma" have been developed in several species to gain information on the potential efficacy of compounds in preventing antigen-induced bronchospasm. Since these models are considerably more complex than the rat passive cutaneous anaphylaxis, compound evaluation in these paradigms generally occupies the terminal step in the pharmacological testing sequence. Models have been developed in small animals such as the rat and guinea pig, utilizing either active or passive sensitization, most frequently with ovalbumin or dinitrophenol–ovalbumin combinations (5–7). Antigen may be presented parenterally for precipitation of systemic anaphylaxis or by aerosol to induce pulmonary anaphylaxis. Pretreatment with an efficacious antiallergic agent would be expected to reduce the severity of the response to antigen. The major advantages of these models are that the principal mediators of pulmonary anaphylaxis have been determined in both species (5–7) and that the sensitized animals are easily and economically prepared in large numbers.

A major disadvantage of these models is that, until recently, they have not allowed for the use of sophisticated technology for measurement of pulmonary mechanics due to the small size of the animals (8). Determination of the effects of antigen or drug–antigen combinations usually involves an indirect assessment of lung compliance rather than a true determination of resistance to air flow in large and small airways.

In contrast, some large animal models of allergic asthma are amenable to measurement of large and small airway resistance (9–11). The principal models used are dogs and rhesus monkeys with a spontaneously occurring hypersensitivity to an antigen prepared from *Ascaris suum* roundworms. The basis of the hypersensitivity is believed to involve neonatal infestation by nematodes, which provokes immunoglobulin E-type antibodies directed against the worm. Although neither dogs nor monkeys are normally subject to infestation with *A. suum* roundworms, a cross-reactivity between common antigenic determinants of the host's natural parasite—*viz.*, *Toxocara canis* in the case of the dog, and *A. suum* is assumed

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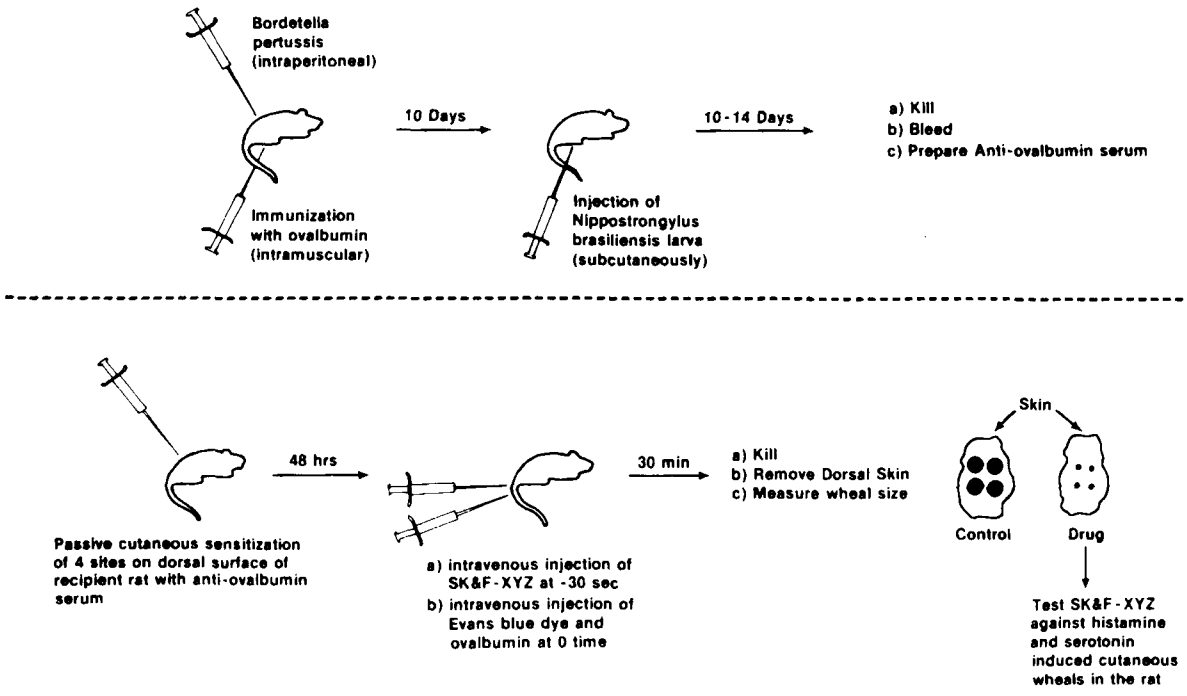

Figure 1—*Schematic of the rat passive cutaneous anaphylaxis model.*

to exist, allowing for the use of this more readily available nematode as an antigen source.

Dogs and rhesus monkeys with hypersensitivity to *Ascaris* antigen are separated from the nonhypersensitive population by a cutaneous testing procedure (Fig. 2). Briefly, the technique consists of injecting Evans blue dye intravenously followed by intracutaneous administration of appropriate dilutions of *Ascaris* antigen. Animals with the appropriate hypersensitivity level respond with wheals and dye infiltration at the antigen injection site. The reaction mechanism is thought to be similar to the rat cutaneous anaphylaxis test in that reaginic antibodies directed against the nematode are firmly fixed to cutaneous mast cells. Subsequent interaction with antigen causes cellular release of the vasoactive substances, leading to an increase in vascular permeability and leakage of dye from the vasculature.

In dogs reared in less than optimal environments, about 50% of adult animals demonstrate a cutaneous hypersensitivity whereas only about 5–10% of the wild adult rhesus monkey population display such hypersensitivity. Animals with appropriate cutaneous sensitivity are subse-

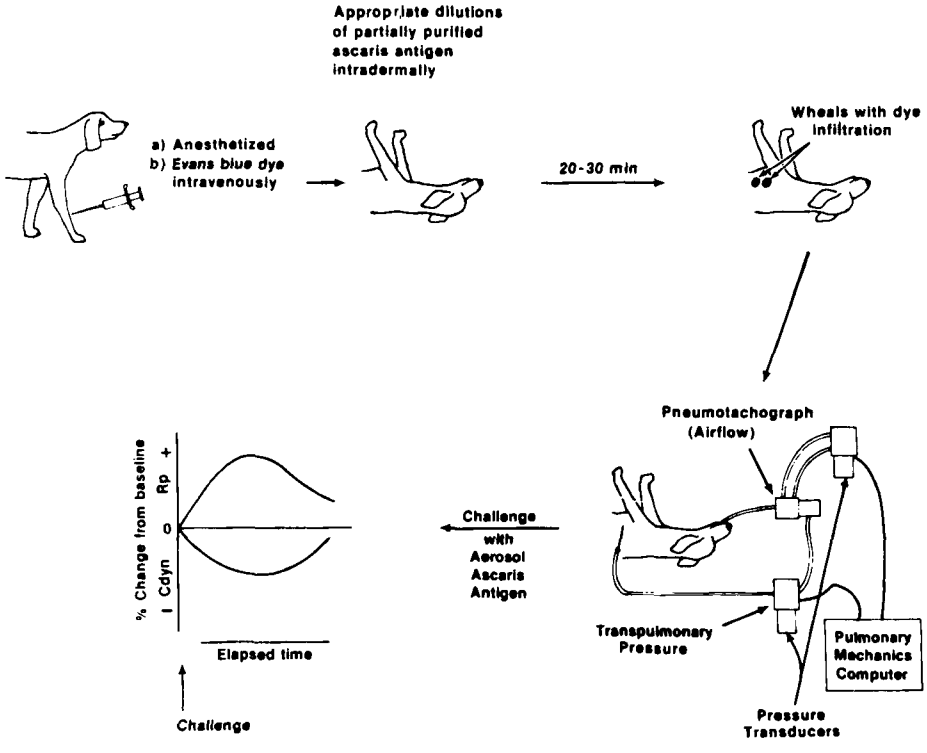

Figure 2—*Schematic of the procedures utilized in the* Ascaris *hypersensitive* in vivo *canine model of allergic asthma. The identical techniques are used for the* Ascaris *hypersensitive rhesus monkey model of allergic asthma.*

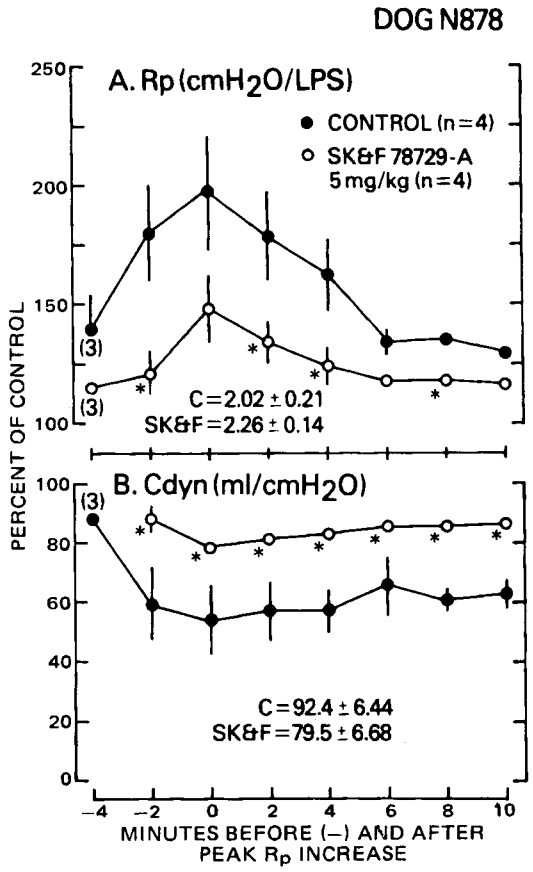

Figure 3—*Effect of I (SK&F 78729-A) on* Ascaris *antigen-induced pulmonary anaphylaxis in the* Ascaris *hypersensitive-canine model of allergic asthma. Compound I was administered (5 mg/kg iv) 30 min prior to seven inhalations of a 1:10 dilution of partially purified* Ascaris *antigen. Rp = pulmonary resistance, Cdyn = dynamic lung compliance, and* n *= number of observations. Numbers in parentheses associated with data points refer to the number of observations for that point only. Each data point signifies the mean* ± SEM. *Baseline values for Rp and Cdyn immediately prior to antigen challenge are illustrated within the graph. (Reprinted, with permission of the publisher, from* J. Pharm. Exp. Ther., *Williams & Wilkins, Baltimore Md.)*

quently challenged with aerosols of the partially purified antigen for determination of airway hypersensitivity. For reasons that are not clear, only a portion of those animals with a cutaneous hypersensitivity demonstrate bronchial responsiveness to the antigen. As with the small animal models, compounds with antiallergic activity are expected to inhibit anaphylaxis when administered prior to antigen provocation. An example of an appropriate pharmacological profile is provided in Figs. 3 and 4 where the antiallergic agent, 5-acetyl-4-hydroxy-3-[1-[(3-amino-4-hydroxyphenyl)amino]ethylidene]-2*H*-pyran-2,6(3*H*)-dione hydrochloride (I) (3), was evaluated against *Ascaris* antigen-induced alterations in pulmonary resistance and dynamic lung compliance in dogs and rhesus monkeys, respectively. Increases in pulmonary resistance and decreases in dynamic lung compliance represent narrowing of large and small airways, respectively. As is apparent, this compound significantly lessened the response in both species.

An alternative to these actively sensitized models is passive sensitization of the airways of nonhypersensitive dogs and monkeys with high titer reaginic serum obtained from hypersensitive donors (10). With monkeys, human serums directed against known allergens may be used because of immunological cross-reactivity between species. Although both systemic and aerosol sensitization may be employed, the latter is most efficient because it requires smaller quantities of serum. While usually successful in the dog, this technique is less applicable to monkeys for unknown reasons, which may relate to the presence or absence of pharmacological airway hyperresponsiveness in the recipient animals.

Rhesus monkeys bronchially hypersensitive to *Ascaris* antigen display an airway hyperresponsiveness to several pharmacological agents including histamine, prostaglandin $F_{2\alpha}$, and cholinergic agonists (12, 13). Dogs do not demonstrate such hyperresponsiveness (14). As noted in the preceding article (1), airway hyperresponsiveness to nonspecific irritants and pharmacological agents is one cardinal clinical feature of the asthmatic. This hyperresponsiveness apparently amplifies the pathophysiological effects of the mediators released on antigen exposure. The demonstration of this phenomenon in monkeys provides an experimental model that may be of utility in uncovering mechanisms underlying this aspect of the disease and perhaps eventually lead to therapeutic agents capable of alleviating the hyperresponsiveness. To date, however, comparatively few investigations have been undertaken with this aspect of the model.

Major disadvantages of large animal models include:

1. Animals with positive bronchial antigen hypersensitivity, particularly rhesus monkeys, are relatively scarce.
2. Antigen challenge can be no more frequent than every 2 weeks. Shorter intervals lead to highly erratic pathophysiological pulmonary responses. Thus, large numbers of animals are required to evaluate reasonable numbers of experimental compounds.
3. Pulmonary responses may be extremely variable, even in the same animal.
4. Large animal models are costly to develop and maintain routinely.
5. No clear insight has yet been gained regarding the biochemical pharmacology underlying the pulmonary pathophysiology. Therefore, unexpected results are difficult to understand.
6. Various pharmacological mechanisms other than inhibition of mediator release can account for a reduction in the response. For example, end-organ antagonism of prominent mediators and smooth muscle bronchodilator activity both reduce response severity.
7. Apparently, humans uniquely suffer from asthma. To our knowledge, no case of allergic asthma in a nonhuman species has been reported. Therefore, while there is presently no alternative, these laboratory models may ultimately prove irrelevant to the clinical syndrome.

For more information regarding these models, the reader is referred to several review articles (9–11, 15).

IN VITRO MODELS

In vitro models of allergic asthma generally occupy an intermediate step in the preclinical pharmacological testing sequence, most often interposed between passive cutaneous anaphylaxis and the more complex *in vivo* models. These systems allow for an assessment of the potential ability of novel molecules to interfere with the antigen-induced release process—the presumed mechanism of action of cromolyn sodium. The most commonly used *in vitro* systems include isolated mast cells (16), blood leukocytes (basophils) (17), and fragment lung tissue (18–21).

A commonly used technique for obtaining a relatively "pure" population of mast cells involves the rat peritoneal cavity. In brief, cells are removed from the peritoneal cavity of actively sensitized rats by a simple washing with a physiological salt solution, and the mast cell population is separated from other peritoneal cell types by centrifugation. Cells are then suspended in an appropriate physiological medium and challenged with antigen to induce histamine release. Preincubation with antiallergic agents is expected to reduce the amount of histamine released on challenge.

Similarly, blood leukocytes (basophils), which also contain histamine and IgE antibody Fc fragment binding sites, may be prepared from actively sensitized animals or allergic human donors, suspended in an artificial medium, and challenged with the appropriate antigen to release the mediators of anaphylaxis. One notable pharmacological peculiarity of human basophils is that immunologically induced mediator release is not susceptible to the inhibitory effect of cromolyn sodium (22). These types of systems are relatively simple and economical, and they provide an essentially homogeneous cell population with which to study antiallergic agents.

The next higher order of complexity in the testing sequence is the *in vitro* fragmented lung model prepared from various species including rats, guinea pigs, dogs, rhesus monkeys, and humans (18–21). Lungs obtained from normal or actively sensitized animals or humans are resected, chopped into small fragments, passively sensitized with high titer reaginic antibody (for those obtained from normal animals), placed in a physiological medium, and then challenged with the appropriate antigen. Many chemical substances can be released on immunological challenge from fragmented lung tissue, and some appear to be species specific. Without regard to species, a partial list of these "mediators" includes: histamine, slow-reacting substance of anaphylaxis, prostaglandins, eosinophil chemotactic factor of anaphylaxis, platelet-activating

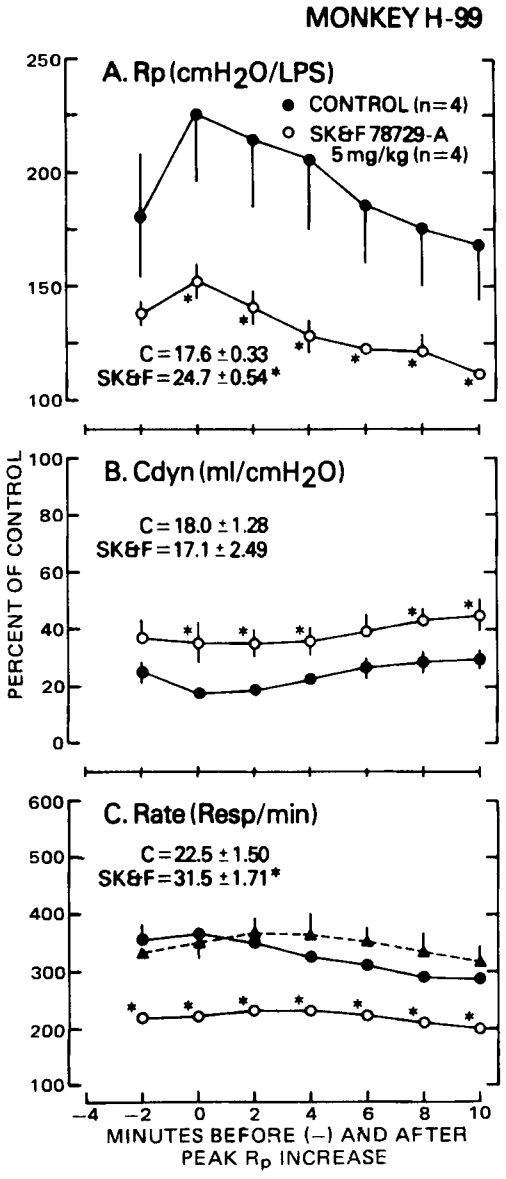

Figure 4—*Effect of I (SK&F 78729-A) on* Ascaris *antigen-induced pulmonary anaphylaxis in the* Ascaris *hypersensitive rhesus monkey model of allergic asthma. Compound I was administered (5 mg/kg iv) 5 min before 15 inhalations of a 1:3 dilution of partially purified* Ascaris *antigen. Rp = pulmonary resistance, and Cdyn = dynamic lung compliance. Baseline values for Rp, Cdyn, and rate immediately prior to antigen challenge are illustrated within the graph. The significant difference in the Rp baseline was not related to the administration of I. Each data point is the mean ± SEM of four separate experiments. (Reprinted, with permission of the publisher, from* J. Pharmacol. Exp. Ther., *Williams & Wilkins, Baltimore, Md.)*

factor, kinins, serotonin, rabbit aorta contracting substance releasing factor, and rabbit aorta contracting substance.

The sheer number of substances released, as well as the rather sophisticated technology required to assay each material, precludes the routine analysis of more than two or three substances. Consequently, many laboratories involved in the search for novel antiallergic agents often measure either histamine or slow-reacting substance of anaphylaxis or both. The selection of these two mediators is based on the observation that much of the body's histamine is sequestered within mast cells, the cells believed to play a major role in the pathophysiological process. Thus, inhibition of histamine release by antiallergic agents provides evidence that the molecule is altering the mast cell release process and, hopefully, that the inhibition will extend to all substances eminating from this cell. Unfortunately, recent experiments indicated that this assumption may not be valid.

The pharmacology of mediator release has been far more extensively studied in these models than in the large *in vivo* models of allergic asthma. It appears well established that agents capable of increasing intracellular levels of cyclic 3′,5′-adenosine monophosphate effectively inhibit histamine and slow-reacting substance of anaphylaxis release from most *in vitro* models (20). Such agents include β-adrenoceptor agonists such as isoproterenol and epinephrine, phosphodiesterase inhibitors including theophylline, and other agonists such as prostaglandins and, perhaps, histamine. Conversely, agents that increase intracellular levels of cyclic 3′,5′-guanosine monophosphate, including α-adrenoceptor and cholinergic stimulants, enhance the immunological release of the mediators (23).

Recently, it was demonstrated that slow-reacting substance of anaphylaxis release can be enhanced by several agents with little or no effect on histamine release, whereas amine release may be augmented or inhibited with no discernible effect on slow-reacting substance of anaphylaxis release (21, 24, 25). These observations suggest the lack of a common release process, at least as regards these two substances; the wider implication is that release of other mediators may also be pharmacologically distinct. Hence, inhibition of histamine and/or slow-reacting substance of anaphylaxis release by novel antiallergic agents cannot be extrapolated to other mediators without reservation. The obscurity of the important mediators of allergic asthma makes this observation unsettling to the preclinical pharmacologist because the inhibitory effects of antiallergic agents on histamine or slow-reacting substance of anaphylaxis release in laboratory models may not translate to an inhibition of the relevant mediators of allergic asthma in humans.

STATUS OF PROPHYLACTIC ANTIALLERGIC AGENTS

Several years of laboratory experimentation with these paradigms have led to the clinical introduction of almost 40 chemically novel antiallergic agents. Unfortunately, only one compound, ketotifen, has been added to the armamentarium and even it is currently limited to use in Switzerland (26). These discouraging clinical results have led to a reevaluation of these models, with attempts to determine which, if any, pharmacological commonalities of the 40 molecules may conceivably account for clinical failures. A number have been identified.

A rapid loss of efficacy as well as tachyphylaxis to the activity of these types of compounds is apparent following oral or parenteral administration in some *in vivo* models or on prolonged preincubation in some *in vitro* models. Although not orally active, cromolyn sodium demonstrates a similar pharmacological profile in these models, yet it remains clinically useful. On this basis, one may be tempted to speculate that such liability in laboratory models does not translate to the clinic and could not account for the clinical failures. However, a thorough determination as to whether this phenomenon occurs when compounds are administered by the aerosol route has not been conducted in laboratory models; thus, aerosol administration of cromolyn sodium may obviate tachyphylaxis and rapid loss of efficacy and prove a caveat for those unwilling to accept compounds not orally active.

Another area of concern is the recent demonstration, as already discussed, that some mediators can be selectively modulated (19, 21, 27). Since the important mediators of the allergic syndrome may be unknown, conceivably these compounds are not inhibiting release of the mediators most relevant to the pathophysiology. Alternatively, the major effects of these compounds may be on mediators of only secondary importance to the bronchospasm. Compounds with such activity may demonstrate marginal to no clinical activity.

Another interesting finding is that these types of molecules appear to be more potent inhibitors of histamine than slow-reacting substance of anaphylaxis release (3). In our experience, the concentration–response curve for these agents on histamine release often lies to the left and above that obtained for slow-reacting substance of anaphylaxis. If, as is often assumed, slow-reacting substance of anaphylaxis is an important mediator of human allergic asthma, a compound at least as potent for slow-reacting substance of anaphylaxis release as for histamine release may prove an interesting clinical candidate. Unfortunately, no new molecules appear to offer this profile.

And, finally, with relatively few exceptions, the newer compounds only partially inhibit the release of histamine and slow-reacting substance of anaphylaxis from most *in vitro* preparations. Frequently, "bell-shaped" concentration–response curves with maximal effects of between 40 and 60% inhibition are characteristic (3). However, cromolyn sodium shares this feature in many models but is clinically active. It is possible to compensate for this disparity by suggesting that cromolyn sodium may be completely efficacious in inhibiting other as yet unknown but important mediators of the human pathophysiology. Again, mechanisms

underlying this partial inhibition remain obscure; however, compounds offering 100% inhibition of both mediators or indeed all mediators would offer a potential clinical advantage.

In summary, as laboratory and clinical experience with both cromolyn sodium and novel antiallergic agents increases, a clearer understanding of the mechanism of action of this class of compounds undoubtedly will emerge. When this time approaches, many problems that concern us today will, hopefully, have fallen away and, perhaps, the design of and search for antiallergic drugs will be considerably less confusing and frustrating than they have been for the past decade.

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Antiasthmatic Drug Therapy and Calcium Ions: Review of Pathogenesis and Role of Calcium

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Abstract □ This paper presents the calcium-dependent pathophysiological features of allergic and nonallergic asthma. Various theories concerning the role of free calcium ions in the pathogenesis of asthma are discussed.

Keyphrases □ Antiallergic drugs—calcium antagonists, inhibition of calcium influx and intracellular calcium movements, inhibition of mediator release □ Calcium-ion dependency—asthma pathophysiology, cyclic adenosine and guanosine monophosphate levels, mediator release, smooth muscle contraction–excitation, mast cell and mucous cell secretion, vagus nerve activation □ Mediator release—antigen dependency, calcium dependency, antiallergic drugs □ Asthma—review of current theories, clinical evaluation, symposium, antiallergic drugs

The principal pathogenetic features of asthma are ultimately calcium-related phenomena: smooth muscle contraction, mast cell chemical mediator secretion, mucous gland secretion, and vagal cholinergic reflex activity. In these cell types, the availability of free calcium ions for excitation–contraction coupling, stimulus–secretion coupling, and nerve impulse conduction determines significantly the smooth muscle contractility, mast cell mediator secretion, mucous gland secretion, and vagus nerve activity. Increased free calcium-ion concentrations might account for heightened smooth muscle contractility and increased mucous gland secretion and perhaps also for an increased mast cell mediator secretion rate and vagal nerve activity.

If the critical pathogenetic pathways in asthma are ultimately related to free calcium-ion availability in smooth muscle, mast cells, mucous glands, and vagus nerve, it follows that effective asthma drug therapy must reduce calcium availability to the essential contractile, secretory, and vagus nerve functions. This concept places altered transmembrane and intracellular calcium movements at the level of final common pathway for the

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