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Progress at the Interface of Inflammation and Asthma

Report of the ALA/ATS Workshop on Future Directions in Asthma Research

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This workshop was held in Leesburg, Virginia, November 10–12, 1993.

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Progress at the Interface of Inflammation and Asthma

Report of an ATS-sponsored Workshop November, 1993

JEFFREY M. DRAZEN and GERARD M. TURINO

Asthma is an inflammatory disorder of unknown cause; it is exceedingly common, with 5–7% of the population in the United States and Europe suffering from this disorder (1–3). We now recognize three distinct components to asthma: the asthma attack, i.e., an episode of airway obstruction that resolves spontaneously or as a result of treatment; hyperresponsiveness of the airways to a variety of provocative stimuli; and persistent, though poorly defined, "inflammation" of the airways. Indeed, there is good reason to believe that the "airway inflammation" of asthma is linked to the biology of airway obstruction and hyperresponsiveness, but the nature of these links has remained elusive (4–7).

MATING INFLAMMATION AND THE CLINICAL ASTHMA PHENOTYPE: THE MEETING OF TWO CULTURES

As we make progress understanding the primal nature of the abnormalities of the airway in asthma, researchers have been working hard to link the identified inflammatory abnormalities to the clinical phenotype that we recognize as asthma. This has not been easy because it requires interactions between two previously very distinct cultures. On the one hand, there are individuals who have been involved in studying the biology of asthma from the perspective of the pulmonary physician and biologist. On the other are individuals whose primary interest has been in various aspects of the inflammatory response and the mechanisms that initiate and perpetuate this response. Only rarely do investigators from these distinct research areas interact.

ATS ASTHMA WORKSHOP-NOVEMBER 1993

As part of its leadership role in working to establish the cause, and possibly a cure, for asthma, the American Thoracic Society sponsored a workshop in the fall of 1993 in which investigators who were working on the biology of asthma met with individuals whose primary interests were in the biology of the inflammatory response. To achieve this goal the conference organizing committee identified a group of individuals whose scientific contributions are considered either outside of or on the edge of asthma research. They also identified a group of scientists whose primary field of endeavor had been in asthma research.

Conference Structure

Four sessions were held, during which various specific components of asthma were considered. First, we considered the mechanisms that initiate and perpetuate the inflammation that is known to exist in the asthmatic airway. Second, effector mechanisms that could link specific aspects of the inflammatory response to alterations in the asthmatic airway were addressed. Third, the role of nonmuscular mechanisms in causing airway obstruction in asthma was discussed. Fourth, we considered the progress that could likely result from dissecting the mechanisms of asthma using the same type of genetic approaches that had been applied by others to understanding other complex disorders. To more widely disseminate the information that was communicated in these sessions, workshop chairs and discussants have prepared summaries of the salient issues that were discussed in each session. These summaries follow this introduction. However, since integration at some level has already occurred, brief précis of each area is presented below.

Lymphocytes and Airway Inflammation in Asthma

There are a number of distinct components of the airway inflammation recognized in asthma, as shown in the Figure. Our current understanding is that lymphocytes bearing the TH_2 phenotype, which respond to stimulation with antigens generally considered among those in the "allergic repertoire," populate the airway in individuals with asthma. When activated, these cells secrete a limited panel of cytokines, including IL-4 and IL-5. These two cytokines are of particular importance because they are thought to drive the differentiation and maturation of mast cells and eosinophils. Because mast cells and eosinophils are known to be present in the asthmatic airway at increased density and because these cells have the capacity to produce effector molecules that could lead to development of the asthmatic phenotype, the presence of these cytokines is thought to be critical to the asthmatic response. Another critical aspect of the presence of the TH_2 cytokine panel is that, in the presence of antigen of the appropriate type, a microenvironment is created that drives the differentiation of B cells with the capacity to produce IgE. Since, it is known that the presence of IgE at elevated levels is associated with the presence of asthma, there is reason to believe that these cytokines are of primal importance to asthma biology. Busse and colleagues (7) summarize the discussions in regard to the role of T and B cells in initiating and perpetuating the asthmatic response.

Effector Mechanisms in Asthma

The presence of primed cells in the airways alone is not enough to initiate an asthmatic response. The cells in the airway must be activated, and the molecules that they secrete or synthesize as a result of this activation lead to many of the phenotypical changes in asthma. Numerous such mechanisms have been identified and may be of importance in asthma biology. Because of the limited time available for discussion at the meeting, only six distinct effector mechanisms were discussed (Table).

We now know that the high-affinity IgE receptor is an important link between the presence of specific antigen in the microenvironment and activation of mast cells and perhaps other cells. Antigen-specific IgE binds to effector cells via specific IgE receptors; when antigen binds an adequate number of these receptors to initiate receptor clustering, signal transduction occurs. The molecular nature of the IgE receptor is clearly defined; it is composed of four chains: an α chain, a β chain, and two γ chains. It is known that the α chain binds IgE, while it is thought that the γ chains are the units that initiate intracellular signal transduction; however, the specific mechanisms of transduction are not established.

The activation of cells via the IgE receptor results in the release of a number of physiologically active biomolecules, including 5-lipoxygenase products and proteases. Five-lipoxygenase products are derived from arachidonic acid released from membrane phospholipids, which are subsequently lipoxygenated. Five-lipoxygenation of arachidonic acid requires the participation of

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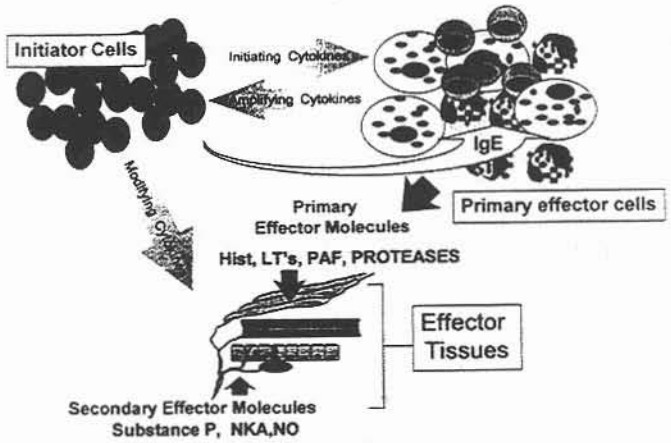

Figure 1. Potential schema of events leading to asthma. The inflammatory events are shown in the top portion of the figure while effector mechanisms are shown below.

an 18kD integral membrane protein found in the perinuclear membranes of a large number of cells. When cells are activated, 5-lipoxygenase is translocated to the perinuclear membrane and initiates its catalytic activity. Among the products that eventually derive from 5-lipoxygenation of arachidonic acid are the cysteinyl leukotrienes; there is abundant evidence that these products are important molecules effecting bronchoconstriction in asthma. Mast cell proteases are also released with cellular activation. Although two major classes of protease, chymase and tryptase, have been designated in humans, there is a wide variety of such proteases in mice, and it seems likely that a broad repertoire may be eventually discovered in humans. The endogenous substrates or the biologic actions of these proteases in asthma are not known. There is a more well-established role for proteases that are present in the microenvironment but are not secreted by activated cells. Among these proteases, neutral endopeptidase (E.C.3.4.24.11 or NEP) is known to be a major enzyme of importance in limiting the biological activity of small peptide mediators such as substance P or neurokinin A. The mechanisms that control the expression of NEP are now in part understood, and their potential role in modulating asthmatic airway responses is becoming better established.

The β-adrenergic receptor and nitric oxide represent two effector mechanisms that are important in modifying the biology of an asthmatic response. The molecular biology of the β-adrenergic receptor is now established in detail, as well as how each of the domains of this receptor interact to transduce a biological effect. An important area of new understanding is how inherited abnormalities of the β-adrenergic receptor may alter its ability to transduce signals. Indeed, there is data suggesting that inheriting alleles that encode for altered forms of the β-adrenergic receptor is associated with a decreased biologic response to receptor stimulation. Among molecules with the capacity to modulate the asthmatic response, nitric oxide has been recently recognized as a potent biologic modifier. The mechanisms of action of nitric oxide as well as the potential for endogenously synthesized nitric oxide to modify asthma biology are just beginning to be understood. Indeed, there are data consistent with both a homeostatic role and a prophlogistic role for nitric oxide. Further information concerning each of these effector systems can be found in the papers presented by Drazen and colleagues and Liggett and colleagues, which follow in this workshop report.

Nonmuscular Airway Obstruction

Smooth muscle constriction can lead to airway obstruction; however, we are just beginning to understand the relevance of nonmuscular airway obstruction. The importance of airway wall remodeling with thickening of the airway wall due to infiltration with inflammatory cells and alteration in the amount and type of collagen deposited in the airway is reflected in the enhanced degree of obstruction that is observed for a given level of smooth muscle activation in the remodeled wall. Another way for the wall to be thickened and hence the lumen obstructed is through the engorgement of the bronchial blood vessels. Indeed, such engorgement could account for a significant component of asthmatic airway narrowing under certain circumstances. The importance of intraluminal fluids, including mucosubstances in asthma, is not well established. Nevertheless, data have shown that the presence of mucus in the airway will obstruct the airway and make it more difficult for individuals to clear secretions from their airways. Boucher and colleagues summarize the discussions in regard to the role of nonmuscular airway obstruction in asthma.

TABLE 1

ASTHMATIC EFFECTOR MECHANISMS CONSIDERED AT THE ATS ASTHMA WORKSHOP*

1. The IgE receptor
2. Five-lipoxygenase products
3. Mast cell proteases
4. Peptidases degrading peptide mediators
5. Nitric oxide
6. The β receptor

* This list is not meant to be exhaustive; rather, these effector mechanisms were chosen because of recent advances in understanding their potential importance in asthma.

Asthma Genetics

Not much definitive is known about asthma genetics, but current data indicate that it is a complex disorder resulting from the actions and interactions of a number of distinct gene products. However, at the same time as our ability to cleanly define the asthma phenotype has improved, more comprehensive linkage maps of the human genome have been created. Improved phenotypical assignment coupled with molecular linkage techniques holds the promise of identifying genetic loci, and perhaps eventually the specific genes, linked to asthma. The paper presented by Bleecker and colleagues summarizes the discussions regarding asthma genetics.

SUMMARY

Most attendees agreed that the workshop achieved its goal of bringing together individuals with a number of distinct scientific approaches to consider new ways of thinking about asthma.

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Mechanisms of Persistent Airway Inflammation in Asthma

A Role For T Cells and T-Cell Products

WILLIAM W. BUSSE, ROBERT L. COFFMAN, ERWIN W. GELFAND, A. B. KAY, and LANNY J. ROSENWASSER

Asthma is characterized by airway inflammation and hyperresponsiveness. In addition to these features, another characteristic marker of the disease is a persistence of these abnormalities. Although many cells participate in the process of asthma, the T lymphocyte has the features and biological properties to not only promote inflammation but also to impart memory. In the following discussion, the role of the T lymphocyte in asthma will be reviewed.

T-CELL IMMUNITY AND INFLAMMATION

T-cell participation in allergic disease and asthma fits well into the general picture of immune regulation (1). The bias now presented is that the CD4+ T cell is the major regulator or organizer of the specific immune system and that it is either a direct effector or at least a stimulator and regulator of most types of immune responses in the body. The concept has emerged that there are two or three major phenotypes of the CD4+ T cells. Although such evidence has been gathered mainly in mouse, there are data to indicate that a similar arrangement exists in humans. The division of T lymphocytes into the TH_1 and TH_2 cell types arose nearly a decade ago in studies by Timothy Mossman and Robert Coffman with clonal T-helper lines from the mouse. It was realized that a large number of clones are not as heterogeneous as originally thought. Consequently, when the cells are classified into patterns of cytokine synthesis, there are many important cytokines, i.e., IL-2, IL-4, IL-5, and interferon-γ, which are made exclusively by one subset or another (2). More recently, another stable phenotype among T-helper clones has been recognized both in mouse and man, which is called TH_0. This subtype is characterized by an ability to generate a large variety of cytokines, including IL-4 and interferon-γ, which are characteristic of either the TH_1 or TH_2 subset.

The importance of T-lymphocyte heterogeneity rests in the fact that virtually all regulatory functions of the T cell are mediated by secreted cytokines (3). Therefore, there is significance in the functional differentiation between the TH_1 and TH_2 cell types. For example, it has been generally noted that strong antibody responses *in vivo* tend to correlate with TH_2 rather than TH_1 responses. In contrast, TH_1 subsets are more capable of mediating classical cell-mediated immune responses to delayed hypersensitivity. This paradigm raises the possibility that disease states may be characterized by distinct subpopulations of the T lymphocyte. Although such evidence has been largely gathered by work in the mouse, recent efforts by Sergio Romaniani and Jan deVries indicate similar patterns exist in man.

It has become apparent that TH_2 cells produce cytokines optimally attuned for stimulation of allergic responses. For example, TH_2 cells make a factor, identified as IL-4, that is a potent inducer of IgE production. Furthermore, it was later recognized that another TH_2 factor, IL-5, is a potent inducer of eosinophilopoiesis and responsible for the regulation of various eosinophil activities. Finally, a combination of factors, IL-3, IL-4, IL-10 and possibly IL-9, provides for optimal *in vitro* growth of mucosal type mast cells.

In contrast, TH_1 cells are primarily responsible for classic delayed hypersensitivity. Moreover, products of the TH_1 type, principally interferon-γ, inhibit or antagonize TH_2 effector functions. IL-4 induces IgE synthesis, and interferon-γ is a strong inhibitor of this process. Such control establishes a model of how IgE can be tightly regulated *in vitro*.

The TH_2 pathway is also involved in regulation of eosinophilia, mast cell activity and, as noted, IgE synthesis (Figure 1). A question fundamental to the division of T cells into two subclassifications is how the development into specific phenotypes is determined. What is the nature of this decision and what signals tell a precursor to became a TH_1 or TH_2 cell type? Evidence exists that IL-4 may act directly on the precursor T cell to induce TH_2 differentiation (3). Two factors have been identified in the mouse that directly induce TH_1 differentiation: interferon and transforming growth factor-beta (TGF-β).

The source of these early cytokines remains unanswered. However, one can speculate that an experimental antigen stimulates an already differentiated TH_2 cell to secrete IL-4 or that a cross-reaction occurs from super antigen-like activity. Second, it is possible that a stimulus may actually deliver a signal to a precursor cell, causing it to make IL-4. This could occur very early during primary stimulation and thus direct cell differentiation. Furthermore, it is possible that IL-4 can come from a third cell type. Once the polarity is established, it appears to be maintained by the ratio of IL-4 to interferon-γ, which may determine this process. For example, if a response starts out as a highly polarized TH_2 response, it can be converted to almost pure TH_1 response by removing IL-4 and adding interferon-γ or interferon-α.

Finally, it is important to stress that the relationship by which interregulation occurs between TH_1 and TH_2, although largely derived from mouse studies, has been similar when evaluated in man. Thus, lymphocyte function is important in regulating immune activity, including infectious responses, allergic reactivity, and as will be discussed later, asthma. The concept that lymphocyte function can be divided into a number of heterogeneous classes is a novel way of showing specific contributions through the generation of their cytokines. Moreover, there is evidence that a specific cytokine profile from these cells can in turn act as an immune regulator of a second cell type. Thus, a concept has emerged by which various subpopulations of lymphocyte can create a very specific immune inflammatory response (4, 5).

T CELL'S ABILITY TO TRANSFER BRONCHIAL HYPERRESPONSIVENESS

The following report will indicate that T-cell sensitization is an important factor in the development of IgE production to a particular antigen and that T-cell subsets are important in establishing the process of airway hyperresponsiveness. Furthermore, the site of T-cell sensitization, i.e., lungs versus systemic, is important in determining bronchial hyperresponsiveness.

The T-cell recognition unit, or T-cell receptor, is composed of α and β chains that recognize antigen presented by a class II peptide (6). In a series of experiments, we have begun to examine whether the β chain, in particular the variable region of the β chain, has a role in directing certain responses to antigen, particularly that of IgE synthesis and airway responsiveness.

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In a mouse model, animals can be sensitized to antigen that is delivered by aerosol. The antibody response to the aerosolized antigen is predominantly IgE and is associated with the development of airway hyperresponsiveness (7). This latter parameter is measured using isolated tracheal smooth muscle and a response to electrical field stimulation. This abnormality can be transferred by intravenously infusing lymphocytes isolated from peribronchial lymph nodes. The lymphocyte transfer is associated with IgE production, immediate cutaneous sensitivity, and airway hyperresponsiveness (8). To further understand the features of this observation, the composition of cells leading to airway hyperresponsiveness was determined by evaluating V-β expression in T cells. T cells isolated from peribronchial lymph nodes and the spleen shared a marked increase in the percentage of V-β 8.1/8.2 positive cells that were responsive to ovalbumin (the sensitizing antigen). In contrast, there was a small increase in cells expressing V-β-2. If V-β 8.1/8.2 cells were isolated and evaluated for their ability to function as T helper cells, IgE production occurred (Table 1). In contrast, if V-β-2 cells were added to V-β 8.1/8.2 T-cells producing IgE, IgE production decreased. V-β 8.1/8.2 cells could also be isolated and transferred to animals. When these cells were transferred, IgE production occurred, as did an increase in airway responsiveness. Thus, a population of T cells can mediate a specific hypersensitivity response, and this activity has specificity for a particular antigen.

In contrast, if animals are sensitized through the cutaneous route instead of the airway, IgE production occurs, but the animals do not develop airway hyperresponsiveness. These data suggest that the presence of IgE alone is not sufficient to cause airway hyperresponsiveness. These experiments also indicate that there is a unique population of V-β T cells that develops in response to aerosolized antigens and that these cells may be critical to the development of antigen-specific IgE responses and airway hyperresponsiveness (9). Thus, the V-β 8.1 T cells can induce manifestations of airway disease that include IgE sensitization and airway hyperresponsiveness.

To expand on these observations, the role of CD8+ cells was evaluated. When passively transferred CD8+ cells from ovalbumin-sensitized animals are evaluated, there is a marked decrease in the IgE response (10). Furthermore, the CD8+ cells from sensitized animals have a high frequency of interferon-γ producing cells. This suggests that interferon-γ production by CD8+ cells may modify the IgE response and may be an important immunomodulator.

To evaluate this latter possibility, interferon-γ was given to antigen-sensitized animals. Intraperitoneal administration of interferon-γ reduced total serum IgE levels but was not antigen-specific. In contrast, nebulized interferon-γ totally blocked antigen-specific IgE responses.

Our data with this mouse model suggest that the transfer of antigen-specific IgE, immediate cutaneous hypersensitivity, and increased airway responsiveness may be mediated, depending on the antigen, by specific V-β expressing T-cell subsets. The question is whether there is a distinct cytokine profile by which these subsets of T lymphocytes modify this response. In preliminary data, only the V-β-8 positive cells, not the V-β-8-negative cells from ovalbumin-sensitized animals, have the capacity to make IL-4. Although not conclusive, these data suggest that subsets of T cells and their local production of cytokines are important in determining local IgE levels and airway hyperresponsiveness.

TABLE 1

TRANSFER OF ANTI-OVA IgE PRODUCTION BY Vβ8.1/8.2 T CELLS

Donor Exposure	Cells Transferred to Recipient	Anti-OVA IgE	Anti-OVA IgG	Anti-OVA IgG1
			EU	
OVA	–	1,348 ± 468	321 ± 39	50 ± 10
PBS	–	< 20	< 20	< 20
OVA	Vβ8.1/8.2	630 ± 54	354 ± 47	< 20
PBS	Vβ8.1/8.2	120 ± 36	103 ± 39	< 20
OVA	Vβ2	< 20	121 ± 5	< 20
OVA	Vβ2+Vβ8.1/8.2	125 ± 15	320 ± 54	< 20

Definition of abbreviations: OVA = ovalbumin; PBS = phosphate-buffered saline.

Two days after completion of the 10-day course (20 min each day) of OVA sensitization through the airways, serum was collected and analyzed for anti-OVA Ig production by ELISA as described (9). Nonimmune control mice were exposed to PBS for the same period of time. In the transfer experiments, Vβ8.1/8.2 and Vβ2 cells prepared from OVA-sensitized mice (8.1/8.2 and Vβ2[OVA]) or from controls (8.1/8.2 and Vβ2[PBS]) were obtained from spleen. Spleens were prepared on day 12, 2 d after completion of the sensitization protocol. 10×10^6 cells per mouse were transferred by intravenous (tail vein) infusion into syngeneic, nonimmunized mice. In cell mixture experiments, an equal number of 10×10^6 Vβ8 and 10×10^6 Vβ2 cells was injected. Immediately after transfer, the recipients were challenged with a single OVA nebulization for 20 min, which by itself did not induce anti-OVA Ig production (10). Five days later, serum was collected and analyzed for IgE, IgG, and IgG1 anti-OVA production by ELISA and expressed as units per milliliter serum. Serum from each animal was analyzed individually in a blinded fashion. The same mice were used for experiments depicted in Figure 1 and Table 2. For each experimental group, mean ± SD are depicted. Statistical analysis was performed by two-tailed Student's *t* test.

T CELL INVOLVEMENT IN ASTHMA

Bronchoscopy with lavage and biopsy is an important tool to sample the lower airway of asthma patients and to compare cell counts in lavage fluids, histology on biopsy, and more importantly, immune regulatory factors that may contribute to altered airway function and responsiveness. This technique has been used to define both the airway and biopsy tissue lymphocyte subtype and the cytokine profile of these cells in patients with asthma, particularly those with active disease. The following discussion will center on how our group has used isolated airway cells, biopsy tissue, and techniques of immunohistochemistry and molecular biology to characterize the immune inflammatory response in asthma. In initial experiments, the airway biopsies had characteristic findings that included the presence of eosinophils and atypical lymphocytes. Eosinophils were expected, but the presence of irregular lymphocytes was unique. To further characterize these cells, they were stained for CD25, a marker of lymphocyte activation; there was an increase in CD25+ cells in patients with asthma compared with those who had allergic rhinitis or normal subjects.

To explain an increase in eosinophils in the airway biopsies of asthma patients, the presence of cytokines that could cause terminal differentiation, prolong survival, and increase their activity, i.e., IL-3, IL-5 and GM-CSF, was evaluated (11). The predominant cytokine found was IL-5, and it appeared to be a product of activated T cells. If such events were operative *in vivo*, they would account for the activation and accumulation of eosinophils in the airway in asthma.

Turning to cells obtained from airway lavage, it is possible to show an increase in CD4+/CD25+ cells that correlates to the patient's bronchial responsiveness and severity of asthma. When these cells were probed for cytokine, IL-5 and IL-4 expression was increased in comparison to IL-2 and interferon-γ (12). This lymphocyte profile is compatible with the TH_2 subpopulation. When similar studies were conducted with patients with pulmonary tuberculosis, the airway lymphocyte cytokine pattern aligned with the TH_1 subpopulation.

To further determine the cell source of the cytokine message, the biopsies were evaluated. Using techniques of double staining, it was possible to identify the cell type that expressed the individual cytokines. The majority of cells that stained positive for IL-4 and IL-5 were CD3+ cells. Macrophages were demon-

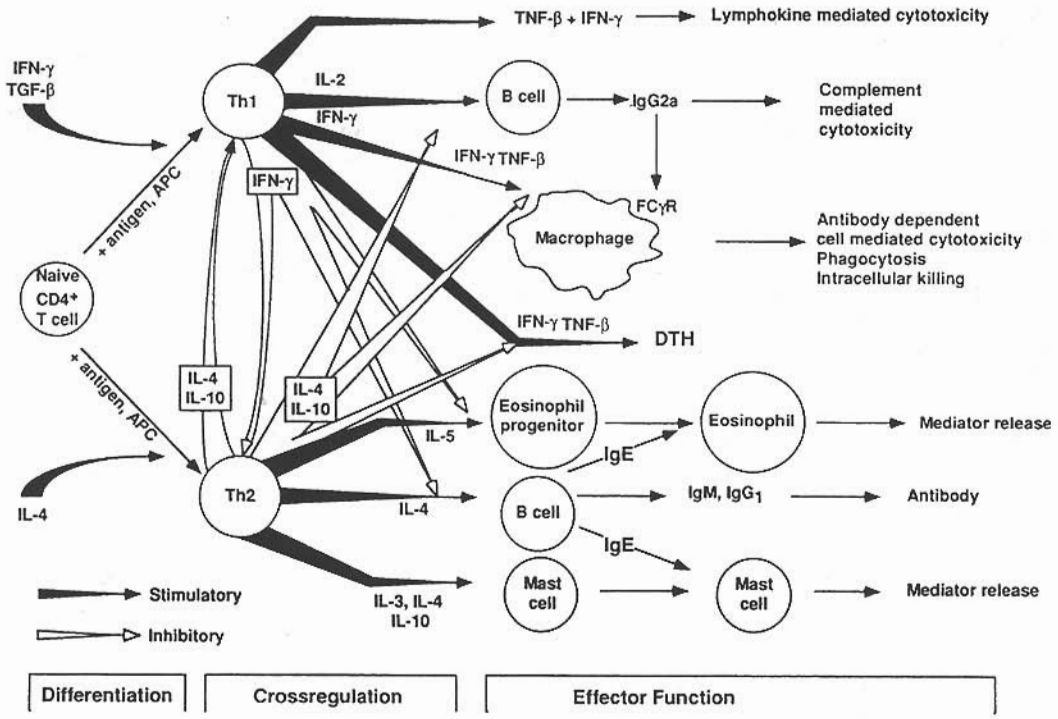

Figure 1. Regulatory interactions between CD4+ T-cell subsets.

strated GM-CSF. A very few eosinophils expressed IL-4, although IL-5 was detectable. Finally, a small percentage of the IL-4 and IL-5 staining cells were mast cells.

To understand how the immune inflammatory events may be regulated, the effect of corticosteroids treatment was evaluated (13). Corticosteroid treatment decreased the number of CD4+/CD25+ cells and also caused a dramatic fall in the number of IL-5–bearing cells. These data suggest that the TH_2 subpopulation of lymphocytes is an important component of asthma and that IL-5 may be a critical cytokine in this disease.

Airway biopsy studies are not possible in patients with severe asthma. To evaluate cellular inflammatory events in these patients, peripheral blood cells were analyzed (14). It was found that CD25+ cells increased in patients admitted to the emergency ward with severe asthma; these values fell after treatment with corticosteroids, and pulmonary function was restored. Furthermore, when peripheral blood lymphocytes were isolated and separated into CD4 and CD8 positive populations, it was found that cytokine expression in peripheral blood cells was localized primarily to the CD4 population. Interestingly, the predominant cytokine found to promote eosinophil survival was GM-CSF.

In summary, in studies that evaluated characteristics of cells in lavage fluid and biopsy, a consistent finding emerged that the CD4 lymphocytes are present, activated (express CD25), and release a cytokine profile associated with the TH_2 phenotype (15). Although the role of other airway cells in this process has not been established, recent data suggest that mast cells may also express IL-4. This finding raises the possibility that mast cells may be important in maintaining the TH_2 phenotype, which can then release a specific cytokine associated with the TH_2 and thus contribute or establish the inflammatory process in the airway in asthma (Figure 2).

GENETIC REGULATION OF T-CELL INFLAMMATION IN ASTHMA

When considering the role of the T cell in asthma and the persistence of abnormalities existent in this disease, it is important to understand that the T cell is capable of generating a memory response, which explains why patients can have recurring symptoms of asthma when exposed to the appropriate stimuli. It is important to consider the effect of the T-cell functions on recognition through MHC T-cell receptor interactions, accumulation at sites of inflammation, and migration through this process. Furthermore, the possibility that subsets of T-helper cells, TH_1 versus TH_2, may be important in this paradigm has emerged as a key feature in asthma. The regulation of this response is likely under the direction of genetic factors.

To understand and identify genetic factors involved in this process, our laboratory has used the airway response in allergic and atopic asthma patients who are sensitive to rodent antigen known as MUS M-1 as an inroad to this process. In these studies, endobronchial challenges with antigen are performed and lavages are completed immediately following challenge and 48 h later.

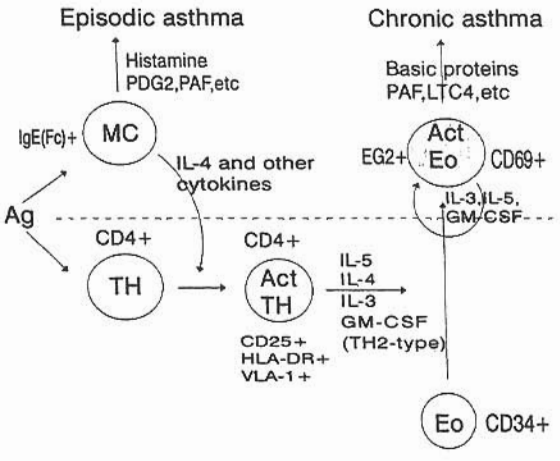

Figure 2. Mechanisms in allergy (using episodic and chronic asthma as an example of allergic inflammation).

Within 48 h of endobronchial challenge, the amount or percentage of airway cells that express IL-2 and IL-4 significantly increases. There is a small increase in interferon-γ that is not statistically significant; this kind of a response is consistent with the profile of a TH_1/TH_2 response discussed previously. It is not known if some of the cytokines may be made exclusively by either the TH_1 or TH_2 subsets. For example, it is not known if IL-2 or IL-4 is made exclusively by either of these subsets. This raises a question as to the basic idea of restricted cytokine profiles, because data indicate that IL-2 may be made by IL-4–producing cells and IL-10 may be made by γ-interferon–producing cells. Therefore, further study of the regulation of cytokine production by various cells is needed. To address this issue, we have begun work to determine IL-4 gene transcription and regulation. From studies of T-cell regulation of IL-2, it has been learned from models of clonal anergy that the IL-2 upstream promoter region is affected in terms of its ability to respond in transcription factors. Therefore, using a similar approach for the IL-4 gene or IL-5 gene should be fruitful and give insights into genetic regulation of the inflammatory response in asthma.

Investigators have begun to localize the site of the promoter gene and related events that regulate IL-4 production. To expand upon this information, studies with Jurkart cell lines were begun and transcription factor analysis ascertained. For these experiments, two oligonucleotides were selected in hopes of eventually identifying sites of abnormality in allergen-specific T-cell clones derived from patients with atopic asthma. Therefore, differences in transcription factors or expression might be identified that represent a point for regulation, or upregulation, of IL-4 production. In this regard, two particular sequences, IL-4/PF-15, were selected. This sequence identifies an area called the P site that conferred responsiveness to PMA stimulation of the IL-4 message. Another oligonucleotide sequence called IL-4/PF-19 was selected that contained the AP-1 consensus sequence, with AP-1 being a common transcription factor thought to be important for IL-4 activation. In stimulated Jurkart cell lines that make IL-4 messenger RNA, some bands were more enhanced. Using this technique, we have begun to analyze what these transcription factors were and the complexity of this issue.

A number of candidate genes for asthma and atopy have been considered. These include MHC, T-cell receptors, IgE molecules, or any of the genetic segments for IgE. We were particularly interested in the cytokine gene cluster on human chromosome 5 and the potential regulation of promoter regions in asthmatic kindreds. The rationale for why these areas may be important is that population studies clearly link IgE to asthma. Airway, immune, and inflammatory cells express the genes for cytokines that regulate IgE synthesis, and many of these cytokine genes are co-regulated on human chromosome 5: IL-3, IL-4, IL-5, IL-9, GM-CSF, and IL-13. Thus, this may be an important candidate locus for asthma.

We have begun by examining 147 complex kindreds of asthma families. To do this, an asthmatic proband is first identified, primarily a child, and then analysis of the rest of the family follows. In this regard, we began to examine about 1,000 base pairs of the upstream promoter region for the IL-4 gene in affected (i.e., patients with asthma) and unaffected members of this asthma kindred. In the initial studies, an approximately 400 base pair area was identified upstream and a product was made with PCR. This was then examined for RFLP polymorphisms among the purified PCR products in both affected and unaffected subjects. We were unable to see any differences in these RFLPs. To expand upon this observation, we used a single-stranded conformational polymorphism PCR technique, followed by sequencing. With this technique, it may be possible to identify a single-point mutation that would not be picked up by RFLP. Promoter regions of all these cytokine genes have been divided into blocks of about 200–350 base pairs. PCR is applied to make a double-stranded DNA, and, after denaturating, they are run on gel after they become single-stranded, from which it is possible to evaluate for the possibility of single mutation change. The 1,000 base pairs upstream of the human versus murine IL-4 promoter region has 87% homology. Therefore, the promoter regions in IL-4 between mouse and human are closer than the coding regions between these two molecules.

We have begun to use these techniques to evaluate kindreds in relationships to IgE synthesis. In subjects with higher levels of IgE, we were able to identify a T-to-C exchange at the position 498 upstream from the transcription start site. Thus far, 16 kindreds have been analyzed, and we have found the presence of polymorphism in six of the 16 kindreds. Whether this is a marker or a transcriptionally active area will require further testing.

Two other polymorphisms have been identified when IL-3 and IL-9 polymorphisms are evaluated. The polymorphisms noted with IL-3 and IL-4 do not correlate with IgE as they do with IL-4. Therefore, it will be necessary to identify whether other factors may be related to these two areas.

We believe that these experiments are important in that they extend the observations beyond the gene product into areas that may be central to the regulation of gene transcription and gene products developed. Whether this will provide novel insight into genetic regulation of atopic asthma will require additional work.

T-CELL INVOLVEMENT IN VIRUS-INDUCED ASTHMA

For many patients with asthma, viral respiratory infections increase symptoms of asthma (16). In clinical investigations, we have demonstrated that rhinovirus experimental infections induce significant changes in the airway response to bronchoconstrictile substances and antigen. For example, rhinovirus increases airway responsiveness and also promotes the likelihood of a late allergic reaction to allergen (17, 18). Enhanced airway responsiveness and the late allergic reaction persist for weeks beyond the viral infection.

With bronchoalveolar lavage and segmental allergen provocation, we have evidence that a rhinovirus infection increases the inflammatory response to antigen in the airway. This conclusion is based on an increase in the cellular infiltrate and eosinophil recruitment to the airway following antigen challenge. These observations raise the possibility that rhinovirus infection promotes both the likelihood and intensity of an allergic inflammatory response. To establish the mechanisms by which this occurs, *in vitro* models have been established to evaluate virus immune cell interactions and how this response can affect inflammation (19).

In these models, peripheral blood mononuclear cells are isolated. Rhinovirus 16 (RV16) is incubated with the mononuclear cell preparation, which contains both lymphocytes and monocytes. Following a 22-h incubation, cells are evaluated by FACS analysis. In these experiments cells are evaluated for the presence of both CD3 and CD69 in hopes of identifying a population of T cells that are activated by RV16. CD69 expression would indicate T-cell activation. In cells incubated with RV16, there is an increased percentage of CD3 cells that express CD69. These observations suggest that the exposure of mononuclear cells to rhinovirus activates a population of T cells. Because the percentage increase of CD3/CD69 cells was large (20%) it is unlikely that this response occurs only via a virus T-lymphocyte interaction. Rather, the possibility exists that an upregulating cytokine is produced, and this product acts on the T cell to cause activation. To address this question, preliminary studies were conducted in which a monocyte suspension was isolated and incubated with

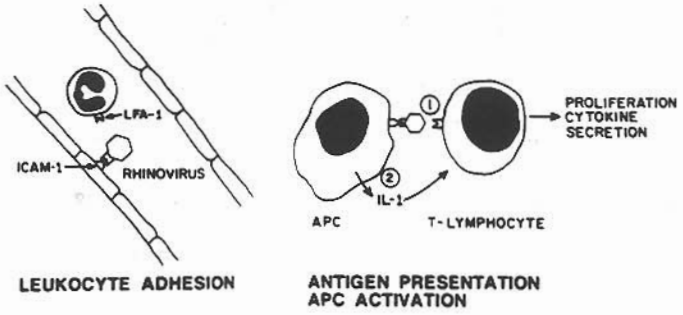

Figure 3. Effect of rhinovirus (RV) on ICAM-1 dependent reactions. ICAM-1 has been shown to be the major receptor for selected strains of human RV. By its binding to ICAM-1, RV could potentially alter several steps in the generation of inflammatory infiltrates. First, antigen presentation has been shown to be dependent on the interaction of ICAM-1 on antigen-presenting cells (APC) with nonspecific adhesion molecules, such as LFA-1, on T lymphocytes. By binding to ICAM-1, RV could potentially inhibit the initial steps in antigen presentation and therefore all the events that subsequently follow (mechanism #1). Alternatively, RV could induce the secretion of IL-1 from APC, which in turn could stimulate the proliferation and secretion of proinflammatory cytokines from antigen-specific T lymphocytes, thereby enhancing the inflammatory response (mechanism #2). ICAM-1 also is located on the vascular endothelium and mediates leukocyte adhesion and transendothelial migration. By binding to ICAM-1, RV could block leukocyte attachment, thereby preventing the development of cell-mediated inflammatory infiltrates. (Reprinted with permission, reference 16.)

RV16. Following RV16 incubation, the cells were evaluated for the expression of mRNA for tumor necrosis factor (TNF-α) and IL-1β. Within 2 h of exposure to RV16, there is increased message for TNF-α; a similar increase response occurred for IL-1β. Following expression of mRNA for these cytokines, protein secretion could be measured in the supernate.

Regulation of this response is important. The major receptor for rhinovirus is ICAM-1 (20, 21). To determine whether the interaction between rhinovirus and immune cells involves the ICAM-1 receptor, the following experiments were performed. Mononuclear cells were incubated with RV16 for 22 h. The cells were then stained with CD54 to identity the cell surface expression of ICAM-1. In cells incubated with RV16, there was a decrease in expression of CD54 on monocytes. This raised the possibility that, because ICAM-1 is occupied by the virus, its expression is diminished. Collectively, these data suggest that rhinovirus interacts with monocytes, possibly through the ICAM-1 receptor, to generate a series of cytokines, i.e., IL-1β and TNF-α. Generation of these proinflammatory cytokines can in turn affect airway tissues.

To extend these studies and determine what effect rhinovirus has on T-cell function, the following experiments were performed. The CD3/CD69 expressing cells were generated by incubation with RV16 and isolated by cell sorting. The isolated cells were cultured for 1 wk and their supernatants collected. These supernatants contained products that promote eosinophil *in vitro* survival. When these supernates were coincubated with monoclonal antibodies, it was determined that the principal eosinophil survival-enhancing activity was related to the presence of interferon-γ.

Thus, it is possible that lymphocytes are activated during incubation with rhinovirus and secrete cytokines, i.e., γ-interferon (Figure 3). Although γ-interferon does not have many proinflammatory activities, such as those described with IL-4 and IL-5, it does affect eosinophil function, including promotion of survival. Furthermore, γ-interferon can augment basophil mediator release. Thus, lymphocyte activation by virus may provide a very different cytokine profile and in this manner selectively enhance inflammation.

SUMMARY

The role of T cells in human allergic inflammation is just beginning to be understood. However, the data presented indicate how the T cell may be a pivotal cell to direct features of allergic inflammation in asthma, how the T cell may be able to transfer hyperresponsiveness, which is a feature of bronchial asthma, what some of the genetic factors are that may determine this process, and how an important precipitant of asthma, viral respiratory infections, may participate in this process. Its cells are isolated from patients with asthma and studied for their ability to generate proinflammatory failure. An expanded understanding of the chronic, persistent nature of asthma will become apparent.

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G-Protein Coupled Receptors, Nitric Oxide, and the IgE Receptor in Asthma

STEPHEN B. LIGGETT, ROBERTO LEVI, and HENRY METZGER

G-protein coupled receptors comprise a superfamily of membrane-bound receptors that signal to the interior of the cell via guanine nucleotide regulatory binding proteins (G-proteins). These receptors have in common an extracellular amino-terminus, seven putative transmembrane spanning domains, three extracellular and three intracellular loops, and a cytoplasmic carboxy-terminus. Over 100 G-protein coupled receptors have been cloned to date, and these receptors appear to be involved in a large number of signaling pathways. These include such diverse processes as yeast mating behavior, slime mold chemotaxis, phototransduction, and odor detection (1). Signal transduction for these receptors involves the binding of a ligand (such as a photon, an odor, a hormone, or an agonist drug), which induces a conformational change in the receptor leading to enhanced affinity for the intracellular portions of the receptor to bind to a G-protein. This complex then alters the activity of an "effector," often an enzyme, which results in a change in the intracellular concentration of a critical substance. The latter is often referred to as a "second messenger" and is typified by cyclic adenosine 3',5' monophosphate (cAMP). In this scenario the ligand can be considered the "first messenger."

G-protein coupled receptors densely populate the pulmonary and cardiovascular system. The focus of this paper is on recent advances in our understanding of β-adrenergic receptors (βAR), with particular emphasis on the relationship between the molecular structure of this receptor and its function. βARs belong to the family of adrenergic receptors that use the endogenous catecholamines epinephrine and norepinephrine (and to a lesser extent, dopamine) as agonists. Nine different adrenergic receptor subtypes have been cloned (2). The α_1ARs, of which there are three subtypes, couple to Gq, resulting in activation of phospholipase C and increases in the second messengers, diacylglycerol and 2, 3 inositol-bisphosphate. All three βAR subtypes (β_1, β_2, and β_3AR) couple to the stimulatory G-protein, Gs, which results in activation of adenylyl cyclase and increases in intracellular cAMP. The α_2ARs primarily couple to the inhibitory G-protein, Gi, which results in decreases in intracellular cAMP. There are at least three subtypes of α_2ARs. (Paradoxically, the α_2ARs can also functionally couple to Gs in some cells [3]). The β_2AR is expressed to some extent in virtually every tissue in the body. In the lung, β_2ARs have been identified in epithelium and smooth muscle of bronchi and bronchioles, submucosal glands, the endothelium of pulmonary arteries, smooth muscle of pulmonary arteries, and alveolar walls. These receptors are also present on immune cells in the lung, including mast cells, macrophages, eosinophils, neutrophils, and lymphocytes. The β_1AR has a somewhat more limited tissue distribution and has been identified primarily in submucosal glands and alveolar walls. The β_1AR is the predominant βAR in the heart; however, β_2AR also populate the heart with concentrated regions of high expression. The distribution of the β_3AR is somewhat controversial (4, 5), but appears to be expressed primarily in brown and white adipose tissue in humans, and is important in thermogenesis and lipolysis. Interestingly, two reports have suggested that β_3AR regulate bronchial smooth muscle tone in the lung in pharmacologic *in vivo* studies, but neither β_3AR protein or mRNA has yet to be identified in pulmonary tissues. However, given that the β_3AR does not undergo agonist-promoted desensitization (6, 7), this receptor may be an as yet untapped target for drugs aimed towards bronchodilation.

The focus on the β_2AR for this asthma workshop is due to several factors. First, of all G-protein coupled receptors, the β_2AR has been the most extensively studied. Paradigms established with this receptor may be applicable to other G-protein coupled receptors as well. Secondly, β_2ARs are the targets for the most rapid-acting and effective bronchodilators used in the treatment of asthma (β-agonists). Third, there is continued controversy surrounding the issue of whether overuse of β-agonists in asthma may result in an adverse outcome. Fourth, β_2AR are also present on other important lung cells involved in asthma (such as mucous glands and epithelial cells), but their role either in the pathophysiology or in the treatment of asthma is poorly understood. Finally, recent genetic polymorphisms of the β_2AR have been delineated in the population (8), which may be the basis of a more severe form of the disease or the basis of the heterogeneity of receptor expression and responses to β-agonists observed clinically.

A schematic representation of the human β_2AR and some of the molecular domains that have been found to be important for receptor function are shown in Figure 1. The ligand binding domains are localized within the α-helical transmembrane spanning regions. These form a binding "pocket," and to some extent each contribute to the environment required for agonist and antagonist binding. The critical regions for agonist binding are within transmembrane regions 3 and 5. Within transmembrane spanning region 5, the hydroxyl groups on two serines at amino acids 204 and 207 appear to form hydrogen bonds with the hydroxyl groups at positions 3 and 4 on the catechol ring. At the other end of the classic catecholamine, the amine group interacts electrostatically with aspartate 113 in transmembrane region 3. In addition, the hydroxyl group on the β carbon of the catecholamine most likely interacts with the hydroxyl group of serine 165 of the fourth transmembrane region (2, 9). The amino-terminus of the receptor appears to be important for proper insertion of receptor into the membrane and also for cellular trafficking of the receptor during agonist promoted densensitization (*see* below). Of particular importance in the above processes are the glycosylation sites located at amino acid positions 6 and 15 of the human receptor. The extracellular loops appear to be important for maintaining overall receptor conformation. The disulfide bonds between the indicated cysteines appear to play a particularly important role in this regard. The first intracellular loop also appears to be important for overall receptor conformation, but this has not been studied in detail.

Coupling between the receptor and Gs occurs at the third intracellular loop. In particular, the extreme amino- and carboxy-terminal regions of this loop, near the membrane, appear to be the key regions for G-protein coupling. There is also a less well-defined role for the second intracellular loop and the proximal portions of the cytoplasmic tail in the coupling process. At position 341 of the human β_2AR is a cysteine that has been shown to be palmitolylated. This post-translational modification is important in the coupling and desensitization process. Although

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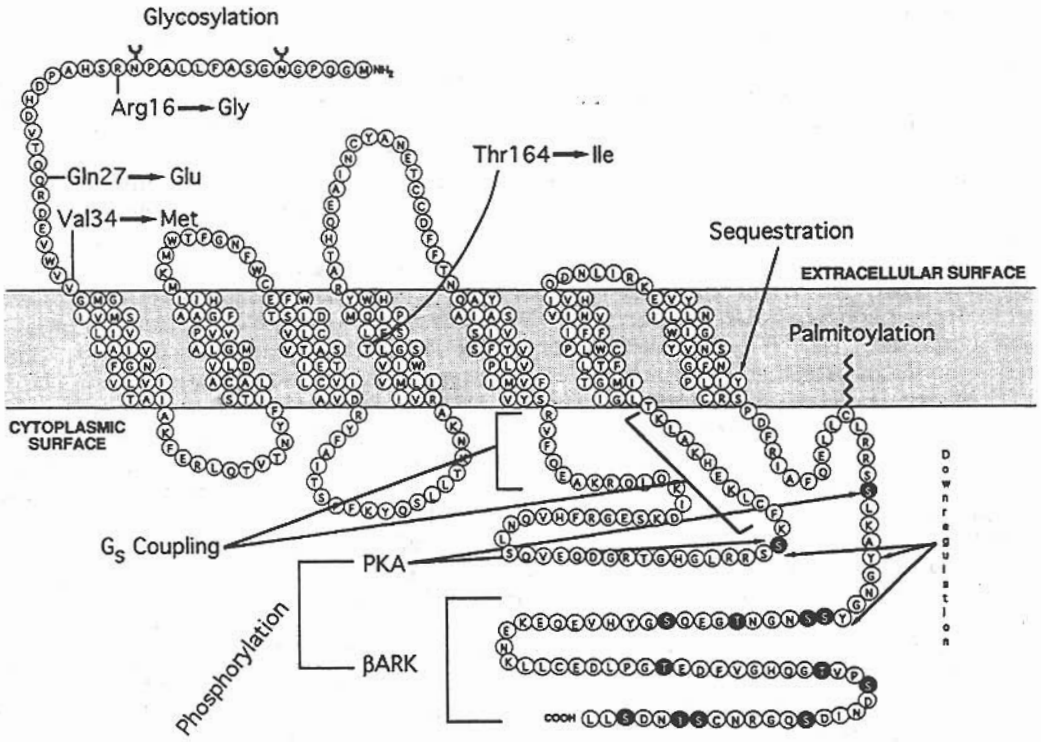

Figure 1. Primary amino acid sequence and proposal membrane topography of the human β_2-adrenergic receptor. Functional domains and polymorphisms, as indicated, are discussed in the text.

this is true for the β_2AR, the palmitolylted cysteine does not subserve the same role for the α_2AR. This underscores the point that the structural features of one G-protein coupled receptor may not serve the same function as does a similar feature in another receptor.

A common finding in many biologic systems is the waning of a response despite the presence of continuous stimulus. This process is variously termed desensitization, tachyphylaxis, tolerance, or refractoriness (7). The β_2AR undergoes desensitization by three distinct mechanisms: phosphorylation, sequestration, and downregulation (Figure 2). Note that desensitization is defined functionally, and any of these mechanisms may be involved

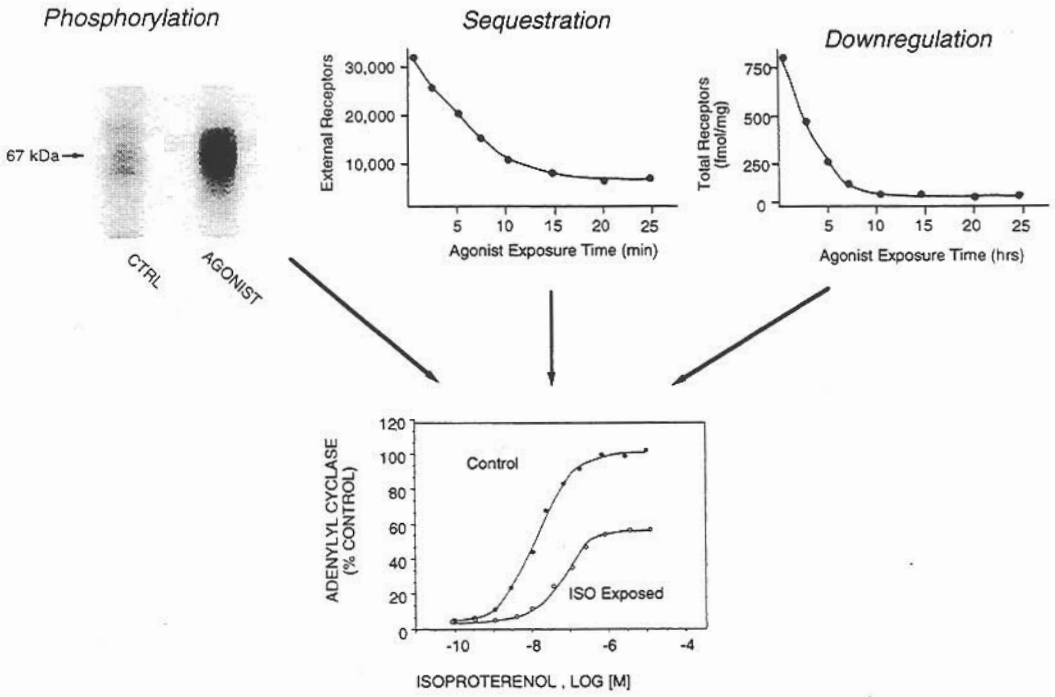

Figure 2. Major mechanisms of agonist-promoted desensitization of the β_2-adrenergic receptor.

in a desensitization process. It is important not to confuse desensitization with downregulation, which as discussed later, is only one mechanism that may be involved in the process. After short-term agonist exposure, β_2AR become phosphorylated at serine and threonine residues in the third intracellular loop and the cytoplasmic tail. Such phosphorylation alters receptor conformation and results in depressed coupling to Gs. The mechanism of this depressed coupling induced by phosphorylation is not fully understood, but apparently the phosphorylated receptor has an enhanced affinity for a molecule termed β-arrestin (analogous to retinal arrestin), and this binding perturbs Gs coupling to the receptor. Phosphatases act to dephosphorylate β_2AR, so under conditions of persistent agonist exposure, an equilibrium between phosphorylated and nonphosphorylated receptors is attained.

The phosphorylation of β_2AR occurs via two protein kinases. Using site-directed mutagenesis, we have delineated by several different models the important molecular determinants for each (see references 2 and 7 for reviews). As shown in Figure 1, two consensus sequences for phosphorylation by the cAMP-dependent protein kinase, also called protein kinase A (PKA), are present in the third intracellular loop and the proximal portion of the cytoplasmic tail. When the serines of the consensus sequence (R-R-S-S) were mutated to alanines and this receptor expressed in Chinese hamster fibroblasts (CHW cells), agonist-promoted desensitization and phosphorylated were blunted. The cytoplasmic tail of the β_2AR has multiple serines and threonines that were thought to be sites for phosphorylation by a cAMP-independent kinase, termed the βAR kinase (βARK). When these serines and threonines were substituted with alanines or glycines and the mutated receptor expressed as above, again short-term, agonist-promoted desensitization and phosphorylation were blunted. When both PKA and βARK sites were removed, short-term desensitization was virtually eliminated. From these and other studies it is clear that PKA and βARK each contribute about equally to the phosphorylation and desensitization of β_2AR during high concentration (high nanomolar to micromolar) exposure to agonist. The PKA-dependent pathway requires only an increase in intracellular cAMP, which can occur during low agonist (subnanomolar) concentration of exposure, while the βARK-mediated pathway is independent of cAMP but requires receptor occupancy.

After receptor occupancy, β_2AR also undergo a process termed sequestration, which is an internalization of the receptor to an intracellular pool (Figure 2). Such sequestration may be the mechanism by which receptors are dephosphorylated and then recycled to the cell surface and may also be the first step in a degradation pathway leading to receptor downregulation. The molecular determinants of sequestration are only now being delineated (Figure 1). Sequestration is independent of phosphorylation and cAMP and has an absolute requirement of agonist occupancy. Downregulation of receptor expression occurs after more prolonged agonist exposure, on the order of hours (Figure 2). Both a decrease in mRNA due to destabilization and receptor protein degradation occur during downregulation. The molecular determinants of downregulation are not well defined, but certain domains of the receptor appear to be necessary, as shown in Figure 1. Downregulation is independent of phosphorylation. Increased intracellular cAMP alone induces some degree of downregulation, but the full process appears to also require agonist occupancy of the receptor.

A number of studies have addressed whether β_2ARs are dysfunctional in asthma, and there appears to be no consensus in this matter. Some studies were performed while patients were receiving drugs (such as corticosteroids or β-agonists) that are known to affect βAR expression or function. In other studies surrogate cells such as circulating lymphocytes were used in lieu of lung tissue. In most studies changes in receptor expression or in functional coupling were assessed in cells without correlations to changes in pulmonary function. It is clear that a defect in β_2AR function is not the primary lesion in asthma. However, because β-agonists are so extensively used in the treatment of asthma, we have attempted to relate our studies on structure/function of the receptor to asthma. A depiction of three possible mechanisms by which the β_2AR may become dysfunctional in asthma is shown in Figure 3. As alluded to earlier, β_2AR undergo desensitization after prolonged agonist exposure by several mechanisms. Under high-dose exposure, such as that which occurs at the neural synapse or with inhaled β-agonist therapy, both βARK- and PKA-mediated phosphorylation occurs. Under low agonist conditions, such as oral β-agonist therapy and elevated systemic catecholamines, only the PKA-mediated process is invoked. Note,

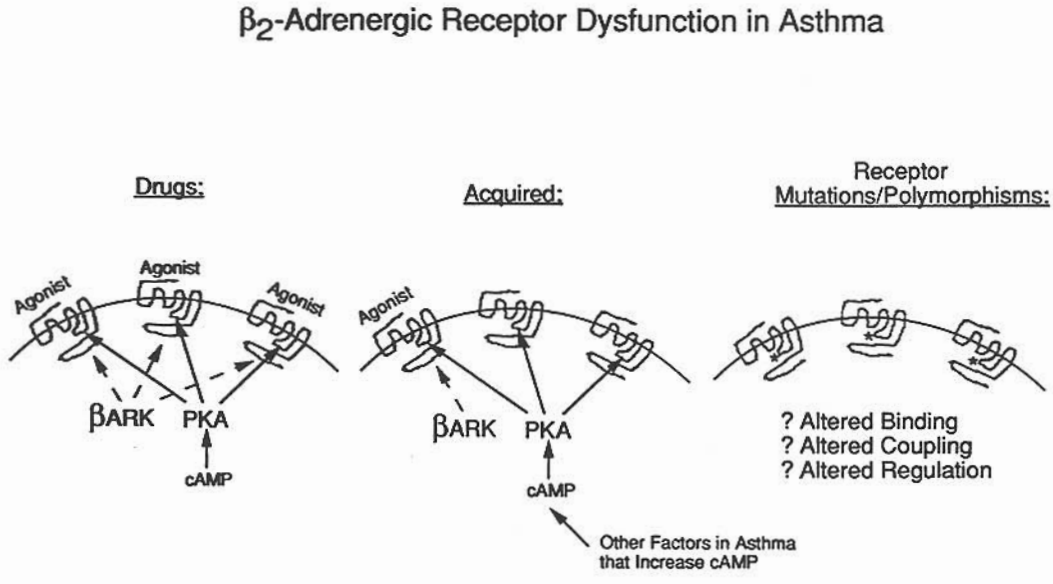

Figure 3. Potential mechanisms by which β_2-adrenergic receptors may be dysfunctional in asthma.

though, that PKA phosphorylates *all* the available β_2AR, even those not occupied by agonist. In fact, any process that leads to increased intracellular cAMP will activate PKA and lead to phosphorylation of all β_2AR in the cell. Thus, even in the absence of agonist, β_2ARs desensitize under such conditions. As discussed earlier, increased cAMP also induces partial downregulation of β_2AR receptor number independent of agonist exposure. Given the large number of mediators that can potentially increase intracellular cAMP in asthma, it is clearly possible that partial desensitization of β_2AR, leading to dysfunctional receptors, can occur in asthma. This latter scenario is what we call acquired dysfunction (Figure 3).

Finally, we have considered the possibility that mutations of the β_2AR may be present in some populations with asthma. To address this, we developed a technique of screening for mutations of the β_2AR coding block using temperature gradient gel electrophoresis (8). Genomic DNA was extracted from peripheral blood samples and PCR was used to amplify five overlapping regions of the coding block. These were then separately annealed to wildtype cDNA and subjected to temperature gradient gel electrophoresis. Any mismatches between patient nucleotide sequence and the wildtype sequence were readily identified, and in those subjects the PCR products were then directly sequenced by the dideoxy method, which identified the specific mutation and the presence of the homozygous or heterozygous state. We found a number of different variations from the wildtype sequence (8), and these are henceforth referred to as polymorphisms. Five differences from the wildtype DNA sequence resulted in no changes in the encoded amino acid sequence. On the other hand, four missense polymorphisms were detected, resulting in the indicated changes in amino acids shown in Figure 1. The most common polymorphism of the β_2AR (up to 50% of the population) is the Arg16→Gly polymorphism, which occurs in the N-terminal region of the receptor. The next most common (~25% prevalence) is a glutamine to glutamic acid substitution, which we term Gln27→Glu and is also localized to the N-terminus of the receptor. Some subjects also had both polymorphisms. We found no difference in the frequency of these two polymorphisms between our two populations of patients with asthma and healthy subjects. A polymorphism that occurs in approximately ~6% of the population is at position 164, where isoleucine is substituted for threonine in transmembrane spanning region 4. This Thr164→Ile polymorphism occurred in three healthy subjects in the heterozygous state and due to this infrequency could not be statistically analyzed. A very rare polymorphism, detected in only one patient, was at amino acid position 34, as indicated. A more complete description and our analysis of β_2AR polymorphisms is published elsewhere (8, 9). While we found no difference in the frequency of the N-terminal polymorphisms between the two groups of subjects, we did find that within the asthmatic group 75% of severe steroid-dependent asthmatics had the Arg16→Gly polymorphism.

To further evaluate the potential for these polymorphisms to play a role in the pathogenesis or treatment of asthma, we mimicked these with site-directed mutagenesis of the wild-type β_2AR cDNA, expressed these receptors in CHW cells, and compared their pharmacologic and biologic properties with that of the wildtype receptor expressed at equal levels. The Thr164→Ile polymorphism displayed a significantly altered ability for the receptor to couple to Gs (9). This altered coupling capacity resulted in a 50% decrease in the ability of the receptor to stimulate adenylyl cyclase as well as a three-fold increase in the EC_{50} for the agonist. The mechanisms by which this polymorphism induces dysfunctional receptor are due to an altered agonist binding affinity (probably due to the effects of this amino acid substitution on the hydroxyl group of the neighboring serine 165 residue), on the ability of the receptor to form the high affinity agonist-receptor-G-protein complex, and on the ability of the receptor to be in the optimal conformation for nonagonist-dependent functions as well (9). The Val34→Met polymorphism was physiologically silent. The N-terminal polymorphisms showed no alteration in the ability of the receptor to couple functionally to Gs, and they each had normal agonist and antagonist binding affinities. However, we found that the ability for these polymorphic forms of the β_2AR to undergo agonist-promoted downregulation was clearly different as compared with the wildtype β_2AR. The Arg16→Gly polymorphism undergoes an enhanced degree of agonist-promoted downregulation after exposure to micromolar concentrations of agonist. In contrast the Gln27→Glu polymorphism appears to be resistant to agonist-promoted downregulation. We are currently studying the mechanisms by which these two polymorphic forms result in different agonist-promoted downregulation. Early evidence suggests that there are differences in receptor protein trafficking of these two polymorphisms. Given that these allelic variants of the β_2AR occur in the normal population, we have begun to consider the possibility that these may be responsible for the heterogeneity of responses to catecholamines that has been observed in healthy subjects. The relevance of these β_2AR polymorphisms may be in that they are providing a contributory role in the clinical course of asthma. Thus they are clinically silent in healthy subjects but become apparent only when there is a need for the receptors either in an endogenous compensatory mechanism associated with asthma or a need for them as pharmacologic targets in the treatment of the disease. We have now begun to more carefully characterize the asthmatic phenotype in asthmatic patients in regard to their response to β-agonists and then assess the relationship between these different phenotypes and β_2AR genotypes.

In summary, the elucidation of the structure/function relationships involved with β_2-adrenergic receptors has provided us with an enhanced understanding of how these receptors transduce their signal, how they are regulated, how they may be manipulated for pharmacologic therapy, how they may become dysfunctional in asthma, and how different polymorphic forms of the receptor may be responsible for different clinical characteristics in patients with asthma.

NITRIC OXIDE

Nitric oxide (NO) is a readily diffusable gas that acts as an intracellular and intercellular signal. In keeping with the interdisciplinary nature of this workshop, the history of the discovery of NO will first be summarized, because it serves to exemplify how work in one field can be applied to other fields with such a ubiquitous and critical signal transduction pathway as that provided by NO.

Our initial interest in NO came from our studies of immediate hypersensitivity reactions as they affect cardiovascular function. An important initial observation was that leukotriene D4 (LTD-4) acted as a vasodilator in preconstricted vessels in an endothelium-dependent manner. Further work indicated that acetylcholine and histamine also caused vasodilatation and that this was potentiated by superoxide dismutase and antagonized by hemoglobin and methylene blue. At that time a substance called endothelium-derived relaxating factor (EDRF) had all these same characteristics. In addition, it was known that nitroglycerin and nitroprusside, potent vasodilators, were metabolized to nitric oxide. Thus, the idea that EDRF was in fact NO was pursued by several laboratories, and ultimately the concept that endothelial cells can produce a gaseous molecule (NO) that is a powerful vasodilator was confirmed (10, 11). The mechanism of NO-induced vasodilatation is by its activation of guanyl cyclase, which catalyzes the intracellular conversion of GTP to cGMP. It was

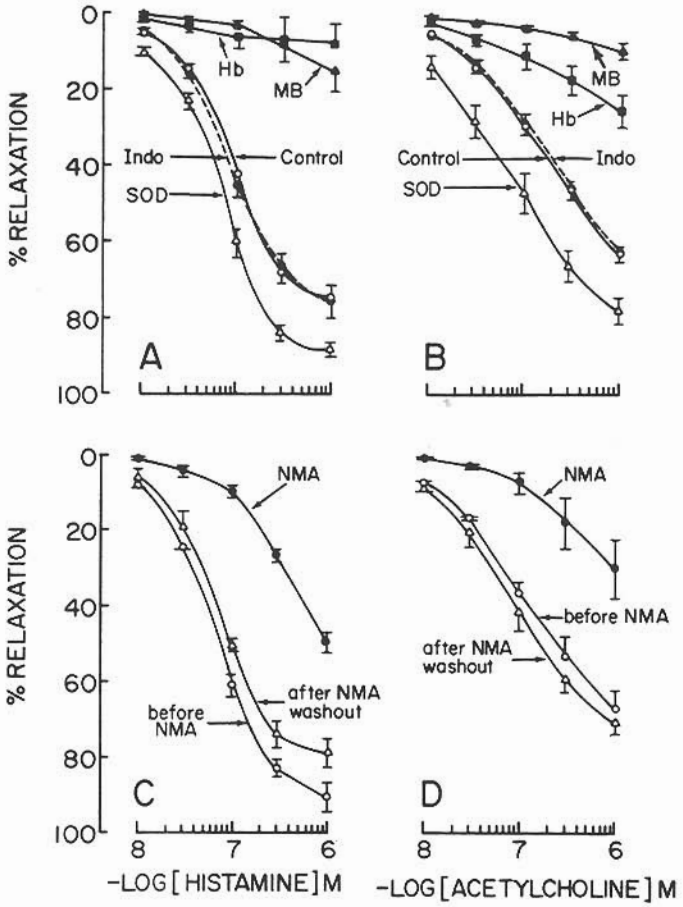

Figure 4. Effects of nitric oxide (NO) and its inhibition by N-methyl arginine (NMA) on vasodilation of guinea pig pulmonary artery. Norepinephrine preconstricted arteries vasodilated in response to histamine and acetylcholine (panels A–D). As shown in panels A and B, the vasodilation was inhibited by hemoglobin (Hb) and methylene blue (MB), unaffected by indomethacin (Indo), and potentiated by superoxide dismutase (SOD). NMA-inhibited relaxation as shown in panels C and D. From reference 12 by permission.

still not understood how NO was synthesized in cells. Soon it became evident that L-arginine was the precursor for NO synthesis and that N^w-methyl-L-arginine (L-N^G monomethyl arginine), variously referred to as NMA or L-NMMA, blocked NO synthesis (12). The effects of NO and its inhibition on vascular smooth muscle are summarized in Figure 4, which is from some of our earlier work (12). As shown, in the presence of NMA the relaxation produced by either histamine or acetylcholine in the pulmonary artery was inhibited in a concentration-dependent manner. Histamine receptors are present on both endothelial cells and vascular smooth muscle cells. Infusion of histamine evoked NO production from endothelial cells and vasodilation occurred as shown. After blockade of NO production, the vasoconstrictive effect of histamine mediated by histamine receptors on the smooth muscle was uncovered. Note that nitroprusside-induced vasodilation is not affected by NMA, because production of NO from nitroprusside (and nitroglycerin) is not dependent on arginine as a precursor.

From these beginnings there has been a marked increase in research on NO pertinent to many organ systems (see references 13–15 for reviews). The focus of this portion of the workshop will be on the potential importance of NO in asthma. Key aspects of the biology of NO as it relates to lung function are summarized in Figure 5. As discussed earlier, NO is synthesized from L-arginine via the enzyme NO synthase (NOS). Two forms of NOS are known. NOS that is independent of Ca^{++}, abbreviated iNOS, is thought to be regulated at the transcriptional level and is also termed the inducible form of NOS. Known inducers of iNOS include tumor necrosis factor α, tumor necrosis factor β, interferon gamma, endotoxins, interleukin-1, and other cytokines. Tissue localization of iNOS includes fibroblasts, macrophages, neutrophils, and vascular smooth muscle. The NOS isoform that is Ca^{++}/calmodulin-dependent is abbreviated cNOS and is also termed the constitutive form. cNOS appears to be localized to epithelial cells, endothelial cells, neurons, platelets, and neutrophils. Stimulation of cNOS occurs through such mediators as bradykinin, histamine, platelet-activating factor, acetylcholine, and several of the leukotrienes. While NOS activity has been documented in airway smooth muscle and pulmonary mast cells, it is not clear which isoform is present in these two tissues.

Given the above, it is obvious that NO has the *potential* to affect a number of cells critical for normal lung function. Airway smooth muscle relaxation, airway neurotransmission, epithelial cell function, immune cell function, vascular smooth muscle cell relaxation, bacteriostasis, and platelet inhibition have all been shown to be modulated by NO. In asthma, the two key questions are: (*1*) Does NO play a role in the pathogenesis of asthma? and (*2*) Can NO or NOS inhibitors be used therapeutically to treat asthma?

Nitric oxide is present in the expired air of healthy individuals (16). Since NO induces bronchial smooth muscle relaxation, one hypothesis has been that NO might be reduced in asthmatic patients. However, expired NO is in fact higher in asthmatic subjects compared with nonasthmatic subjects (17). In addition, airway epithelial NOS is higher in asthmatic subjects compared with healthy subjects (18). This implies that NO may increase in asthma as a compensatory response to other factors, such as those that cause bronchoconstriction or inflammation. As shown in Figure 5, a number of bronchoconstrictor substances and inflammatory mediators do in fact stimulate either cNOS or iNOS. However, the potential role for NO and NOS in asthma is further complicated by their diverse sites of action and tissue distribution, respectively. For example, if vascular engorgement is important in the pathogenesis of asthma, then elevated NO might exacerbate bronchial obstruction because NO relaxes vascular smooth muscle. In addition, elevated NO may result in elevated NO reaction products, such as those that occur with superoxide. These products, such as peroxynitrite, may result in airway damage if in excess. Thus, it is not clear whether elevated NO is part of the primary pathologic process in asthma or is a compensatory response. Concerning the use of NO for treating asthma, there are few data at this time, but protocols are underway in several centers.

The problems associated with delineating the role of NO in asthma and determining whether modulating NO is a feasible therapeutic modality needs to be approached along several fronts. Ideally, the cellular localization of iNOS and cNOS needs to be precisely mapped in human lung, preferably by *in situ* hybridization. As animal models of asthma continue to be developed, such localization needs to be carried out in the lungs of these animals as well since they are amenable to more invasive physiologic studies. Further molecular details of NOS structure/function need to be delineated, and the mechanisms of how the expression of the two enzymes are regulated needs to be further defined. More details on how NO, once generated, diffuses to effector cells is also critical, as well as a better understanding of the pertinent *in vivo* conditions that affect the stability of NO or its subsequent products. Finally, NOS gene ablation and over-

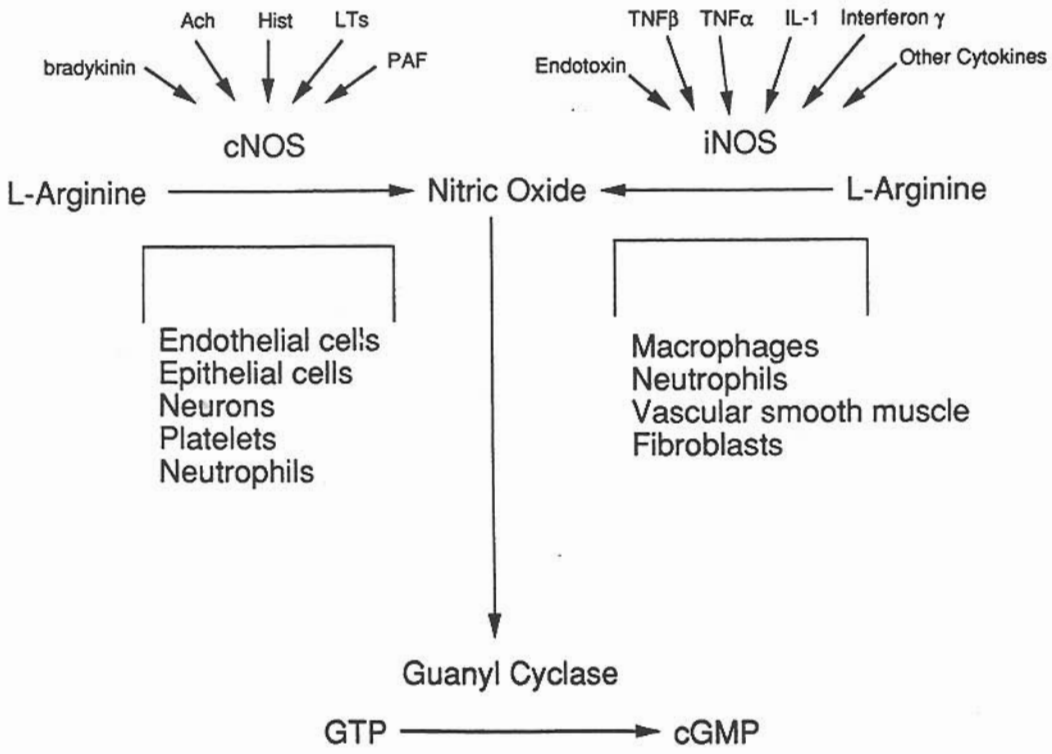

Figure 5. Key aspects of nitric oxide synthesis and function as it relates to lung function.

expression in mice using lung cell-specific targeting would provide invaluable insight into the role of NO in lung function in general and its potential role in the pathogenesis and treatment in asthma.

THE HIGH AFFINITY IgE RECEPTOR

Experimental models now exist that will allow testing of the pathogenesis of asthma and related disorders. In addition to the mice that lack mast cells, mice in which the receptor with high affinity for IgE has been "knocked out" have recently been prepared by Dombrowicz and colleagues (19). The latter animals have otherwise normal mast cell development and IgE production but are unable to undergo a (passive) anaphylactic reaction when suitably tested. The availability of these models and exciting progress on defining the function of the high affinity receptor at a molecular level make it increasingly likely that soon one will be able to test whether this knowledge can be used therapeutically.

In this workshop our knowledge (reviewed in references 20–23 in detail) of the structure and function of the high-affinity receptor for IgE has been related to several strategies based on targeting the receptor to modulate allergic responses. Antigen stimulation of the degranulation of mast cells or basophils via the IgE receptor involves two discrete steps. The first involves the binding of IgE to the receptor. This binding has a very high affinity, largely because the IgE dissociates from the receptor very slowly. The practical implication is that one can hope to prevent the binding of IgE ("sensitization"), but for all practical purposes one cannot expect to be able to reverse binding once it occurs. As far as is known, this initial binding does not in anyway perturb the cell. Indeed, conceptually one should consider the IgE as an exogenously synthesized portion of the receptor that confers antigen specificity to the receptor. The subsequent triggering of the receptor occurs as a consequence of the cell-bound immunoglobin being aggregated by a multivalent antigen or allergen. This mechanism by which the receptor becomes activated is the same one used by some of the other principal receptors of the immune system and is quite different than those used by other types of receptors, such as G-protein coupled receptors. In the latter instance, activation may involve a critical conformational alteration of the receptor induced by ligand binding.

A schematic of the receptor's structure is shown in Figure 6. The receptor's α chain is a transmembrane protein whose external portion is composed of two immunoglobulin-like domains. It is this region that contains the binding site for IgE. The α is associated with a β chain that traverses the membrane four times, with a disulfide-linked dimer of γ chains. The latter type of chain is now known to be a subunit shared by several other so-called Fc receptors and plays a central role in the ability of the receptor to initiate several different biochemical cascades. A related chain —the ζ chain—plays an analogous role in the function of the antigen-specific receptor on T lymphocytes.

An obvious way that one could interfere with the allergic response would be to interfere with the binding of the IgE to the α chain. One way to look for an inhibitor is to develop a rapid assay and to test a large number of unselected compounds in the hope of getting a "leader" compound whose structure could then be systematically modified to develop an inhibitor that could be used *in vivo*. That approach is in fact being used by several pharmaceutical companies. Alternatively, one could try to first define the site(s) of interaction on the receptor's α chain and on the Fc region of the IgE and use that knowledge to rationally develop an inhibitor. Regardless of whether one is trying to define the site on the receptor or on the IgE, the approaches are very similar. One strategy has been to define regions smaller than the entire external portion of the α chain that retain binding activity. Nowadays this is most readily done through site-directed mutagenesis, but one can also do it by preparing peptides representing different linear sequences and see if they will in-

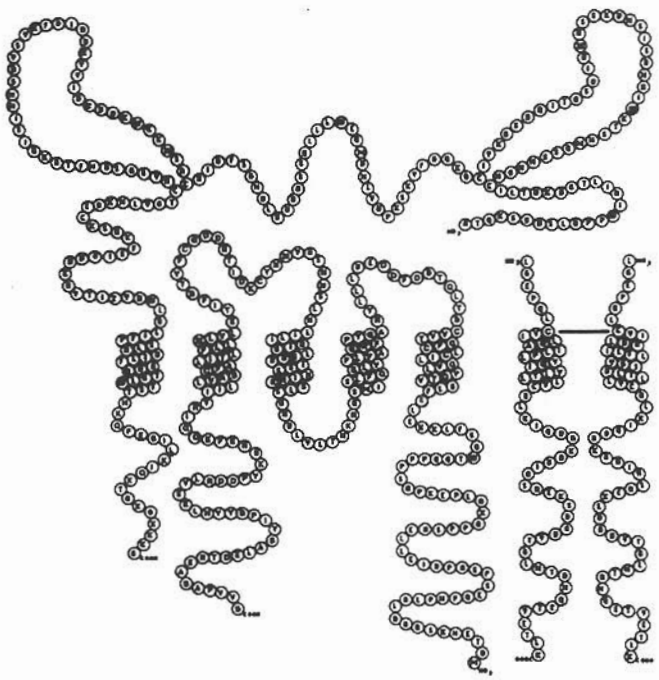

Figure 6. Primary amino acid sequence and proposed membrane topography of the high-affinity IgE receptor. The four subunits are arranged for illustrative purposes from left to right. The left continguous stretch of residues is the α subunit, which traverses the membrane once. The β subunit is in the middle of the figure and traverses the membrane four times. Two γ subunits linked by a disulphide bond are on the right side of the figure.

hibit binding. Alternatively, one can make antibodies to the receptor, to the IgE, or to peptides representing specific sequences on either molecule. By testing which antibodies inhibit binding, one can begin to define the critical regions. All of these methods have been applied to this problem, and all have had some partial success. However, so far no single small region that can be defined by a short sequence has been demonstrated to be an effective inhibitor. We refer to this approach as a means of *divining* the combining site. To really *define* the sites of interaction, only the methods of x-ray diffraction or nuclear magnetic resonance will suffice. Only at the level of resolution that these methods are capable of providing will one get information that is sufficiently detailed to provide the basis for synthesizing a designer drug. Recombinant expression technology now allows one to produce sufficient amounts of proteins for using these methods. Furthermore, since the carbohydrate on the receptor and the IgE does not appear to be critical for binding but can interfere with making crystals from the proteins, one can use recombinant expression approaches to selectively remove the sites that become glycosylated. Several groups are pursuing this approach. Although one can safely predict that eventually this approach will be fruitful, it may take some time. One group has been using this approach to try to make crystals suitable for analysis by x-ray diffraction from Fc receptors for IgG, and after over 5 yr they have yet to be successful.

An alternative approach is to try to define some of the earliest events mediated by the receptor for IgE and learn how to interfere with them. The reason one would like to target the proximal events is that these are most likely to be specific for the receptor. Because the latter is found only on mast cells, basophils, and related cells, there is a greater possibility that interfering with the early steps will not result in unwanted side effects.

One strategy is to focus on the mechanistic role of the receptor itself, because that is where the action begins. For example, one could attempt to define what portion of the receptor is involved in the activation step by making selective mutations in the receptor. The difficulty in assessing the consequences of these changes is to find an appropriate cell that lacks the receptor but retains the immediate post-receptor components that will respond to aggregation of the transfected receptors. Several years ago we performed such studies and found that an old war horse of immunology, the P815 mastocytoma cells (a favorite target for those studying cell mediated cytotoxicity), were suitable for this purpose. We examined the capacity of transfected wild-type and various mutant receptors to mediate a receptor-initiated rise in intracellular Ca^{2+}, in hydrolysis of phosphoinositides, and in phosphorylation of protein tyrosines. A principal result of these studies was that regardless of whether a particular mutant affected a function or not, all three of the functions monitored were affected in unison, suggesting they are interrelated. Certain modifications produced no effect. For example, mutants in which we had truncated the cytoplasmic domain of the α chain or the amino-terminal end of the β-chain behaved like the wild-type receptor. On the other hand, those in which the cytoplasmic domain of the γ chains or the carboxy-terminal portion of the β chain had been truncated showed absent or sharply diminished function. Notably, these latter two portions of the receptor each contain an amino acid sequence "motif" that had been implicated as one that is common to many receptors that share functional characteristics with the high-affinity receptor for IgE.

A number of investigators have taken a direct approach and prepared chimeric proteins containing an irrelevant extracellular and transmembrane domain fused to a cytoplasmic domain containing one or more of the tyrosine activation motifs. Remarkably, they found that by aggregating these minimal kinds of receptors, for example, with a suitable antibody to the extracellular portion, they could stimulate cellular responses closely resembling those initiated by the complete receptor. Although one cannot assume that the remaining portions of the native receptors are irrelevant, it does give one a powerful tool by which to investigate the early receptor-mediated events.

It was Reuben Siraganian's laboratory at the NIH that in recent years provided the first convincing data that phosphorylation of protein tyrosines was one of the earliest signals mediated by the high-affinity receptor for IgE (24). They initially focused on a 72 kDa component, but further studies by them and others revealed that a substantial number of other cellular components undergo similar modification as an early consequence of receptor aggregation. An example of this phenomenon is depicted in Figure 7. In this case we have used electrophoresis in sodium dodecyl sulfate on a polyacrylamide gel to analyze which proteins become phosphorylated. This is done by reacting the gel with an antibody to phosphotyrosine. The bound anti-phosphotyrosine is then detected by enhanced chemiluminescence. If one precipitates the receptor from an extract of solubilized cells, one can observe an aggregation-dependent tyrosine phosphorylation of the β and γ chains of the receptor.

Because this latter phenomenon is an early event, we have focused on it and tried to answer some straightforward but fundamental questions. For example, we still do not know exactly what aggregation actually does (20). Nor do we know how long after aggregation the receptor remains active. To answer this latter question, we have used chemically cross-linked small oligomers of IgE. These are added to the cells, and shortly thereafter a large excess of monomeric IgE is added to prevent further clustering. Because of the slow dissociation of IgE from the receptors, those receptors that were initially clustered by binding the oligomers remain clustered. The receptors as well as several other cellular components remain phosphorylated over a prolonged period. Ad-

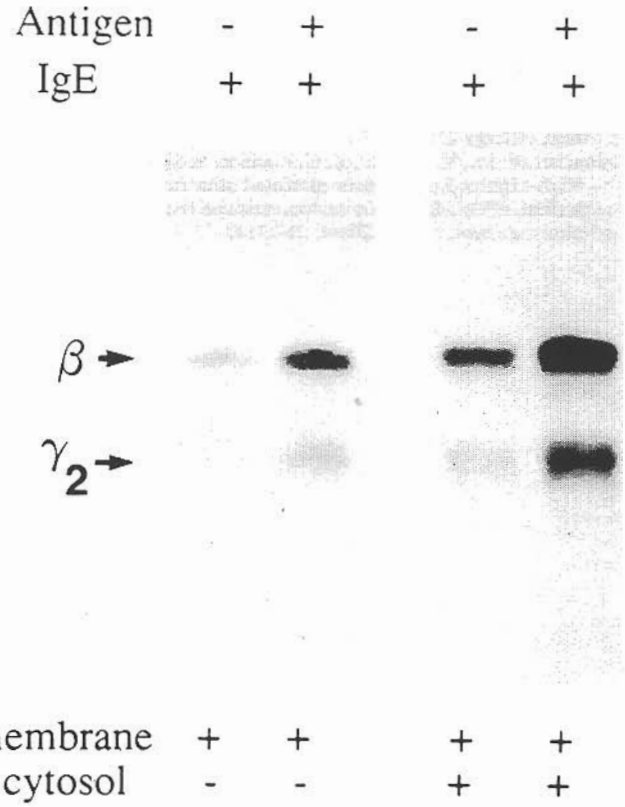

Figure 7. Phosphorylation of tyrosines on β and γ subunits of IgE initiated by aggregation. From reference 20 by permission.

ditional experiments showed that this state of phosphorylation represents a dynamic equilibrium state, with the proteins apparently being constantly phosphorylated and dephosphorylated. Basically, these experiments demonstrate that the cell remains responsive to the aggregated receptors over a prolonged period of time. Furthermore, when we examined secretion by the cells using a similar protocol, it was apparent that not only the early events but the whole train of events initiated by receptor aggregation continued to be stimulated as long as the receptors remained aggregated. With the help of some mathematicians, we are using the data from such studies to test some simple models to see whether they fit the quantitative data developed experimentally. If the mechanism by which the receptor initiates an event such as protein phosphorylation was relatively simple, then one might expect to be able to detect at least some of the earliest events on broken cell preparations. Recently, we were successful for the first time in demonstrating such a phenomenon on isolated membranes. Because such preparations are so much simpler than a living cell, it should provide a powerful tool by which to investigate further the precise mechanism by which receptors trigger the cellular responses.

Another question that is being explored by several laboratories is the nature of the proteins that may be associated with the receptor in the plasma membrane and whose coaggregation with the receptor may provide the initial signal. Here, too, there is rapid progress. At least two discrete protein tyrosine kinases have been identified, principally using rodent cells. One is a src-like kinase, of which there are by now numerous varieties. The one used by the high-affinity receptor for IgE in rat cells appears to be *lyn*; the corresponding enzyme used in mouse cells appears to be *yes*. A second type of kinase is related to or identical with the kinase *syk*, which has been strongly implicated as critical to signaling by the B-lymphocyte antigen receptor; a closely related kinase called *zap* appears to play a similar role in T lymphocytes.

In closing, what we know about the central role of the receptor with high affinity for IgE in allergic responses indicates that it is a promising target on which to focus attempts to modify these responses. We are rapidly accumulating considerable molecular detail about the interaction of IgE with the receptor, the structure of the receptor, and the immediate "post-receptor" components that are either pre-associated with the unaggregated receptor or that become associated with the receptor on aggregation. It is not overly optimistic to anticipate that inhibitors of both the binding of IgE and the early signals generated by the receptor will become available before too long.

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Inflammatory Effector Mechanisms in Asthma

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Asthma is an inflammatory condition. As such, one mechanism that could lead to the physiologic alterations that characterize asthma is the action of mediators or enzymes released from inflammatory cells on target tissues. In this regard we consider three distinct effector systems of potential importance to the asthmatic response: the 5-lipoxygenase system, mast cell proteases, and peptide cleavage by neutral metallo-endopeptidase. Each will be reviewed separately.

5-LIPOXYGENASE PATHWAY

The original discovery of a slow-reacting substance was that of a smooth muscle contractile activity distinct from histamine; it was distinguished from histamine on the basis that its effects were slow in onset and prolonged in duration (1). The subsequent isolation and elucidation of the structure of slow-reacting substance as a mixture of the cysteinyl leukotrienes (LT), now known as LTC_4, D_4, and E_4, resulted in a major advance in understanding the biology of these agents (2). It is now known that arachidonic acid is cleaved from cell membranes, likely by cytosolic phospholipase A_2; the arachidonic acid so liberated interacts with the enzyme 5-lipoxygenase. Five-lipoxygenase is a 78-kilodalton enzyme that has a non-heme iron as a required cofactor and also requires ATP and calcium for activity. This enzyme catalyzes two reactions on nuclear cell membranes. These reactions include the addition of molecular oxygen to arachidonic acid to form 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and the subsequent dehydration to form the unstable epoxide intermediate known as leukotriene A_4. For 5-lipoxygenase to function, it must be moved from a soluble compartment to the nuclear cell membrane. This translocation is followed by activation by a protein known as the 5-lipoxygenase-activating protein or FLAP. FLAP is an 18-kilodalton, 161-amino acid membrane protein (3) (Figure 1). The mature protein putatively contains three transmembrane domains. Through transfection experiments, it has been shown that the expression of both 5-lipoxygenase and FLAP is required for cells to produce leukotrienes (4). Site-directed and deletion mutagenesis studies have demonstrated that amino acids in the first loop separating the proposed first and second transmembrane domains are critical for the binding of FLAP inhibitors MK-886 or MK-591 (5).

Immunoelectron micrographic studies have shown that both FLAP and 5-lipoxygenase are located on the nuclear and perinuclear membranes of human leukocytes (6). These studies have shown that 5-lipoxygenase colocalizes with the FLAP in activated human polymorphonuclear leukocytes, where about 65% of the activated 5-lipoxygenase is associated with the inner nuclear membrane. These data suggest that the arachidonic acid substrate for leukotriene synthesis is derived from nuclear and perinuclear membranes and is selectively made available to 5-lipoxygenase by FLAP to form LTA_4 in the proximity of the nucleus. The LTA_4 so produced can be transported out of the cell or converted to leukotriene B_4 by the cystolic enzyme, LTA_4 hydrolase. This is the enzymatically preferred reaction in neutrophils. This same enzyme can also function as a zinc-independent amino peptidase and is widely distributed in many cell types.

In cell types such as mast cells and eosinophils, where the enzyme leukotriene C_4 synthase and glutathione are available, glutathione is enzymatically conjugated at the C_6 position of leukotriene A_4 to form the molecule known as leukotriene C_4. Leukotriene C_4 is cleaved to leukotriene D_4 by the action of gamma-glutamyl transpeptidase and further to leukotriene E_4 by a variety of dipeptidases (Figure 2). Leukotriene D_4 and E_4 share a common receptor, now referred to as the LTD_4 receptor type 1 (CL_1) which, when stimulated, can cause bronchospasm, increased vascular permeability, and mucus secretion. This receptor, however, has so far eluded molecular isolation.

Under appropriate activation conditions, leukotriene B_4 or the cysteinyl leukotrienes can be synthesized from arachidonic acid. Although these molecules are known to have a variety of biologic activities, studies in a variety of human asthma models, as well as spontaneous asthma, have suggested that products created as a result of activation of this pathway are associated with many of the clinical changes in asthma.

MAST CELL PROTEASES

Mast cells are IgE receptor-bearing, effector cells of the immune system that play a pivotal role in allergic and inflammatory reactions. As much as 70% of the weight of a mast cell consists of proteases that are enzymatically active at neutral pH. Since mast cells contain at least 10-fold more neutral protease than polymorphonuclear leukocytes, they are the major source of neutral protease in most connective tissues. Until 5 yr ago, it was assumed that mast cells expressed only one or two serine proteases. However, recent evidence has been obtained in the mouse and human that indicates mast cells can express a complex array of proteases. Proteases have been purified from *in vivo*- and *in vitro*-differentiated mast cells sufficiently to deduce their N-terminal amino acid sequences (7, 8). Based on the obtained amino acid sequence data, libraries have been screened with relevant synthetic oligonucleotides to obtain cDNAs, and subsequently, the genes that encode seven distinct serine proteases designated mouse mast cell protease mMCP-1 to mMCP-7, and the exopeptidase designated mouse mast cell carboxypeptidase A (mMC-CPA).

Guided by differences in their tissue distribution, histochemistry, morphology, and functional response to different secretagogues, it has been concluded that a number of types of mast cells exist in tissues. In the mouse, connective tissue mast cells are found predominantly in the loose connective tissues and submucosa, whereas mucosal mast cells are found in increased numbers in helminth-infected intestines. Recent data indicate that mast cell heterogeneity in the mouse is more complex than previously thought. One reason mast cell heterogeneity was not fully appreciated in the mouse and in other species was that polyclonal antibodies raised against the proteases cannot distinguish one homologous protease from another. More specific antibodies have been derived recently employing as antigens synthetic peptides that correspond to unique sequences within mMCP-2, mMCP-5, mMCP-7, and mMC-CPA. Using these antibodies and gene-specific cDNA probes, it was recently discovered that the

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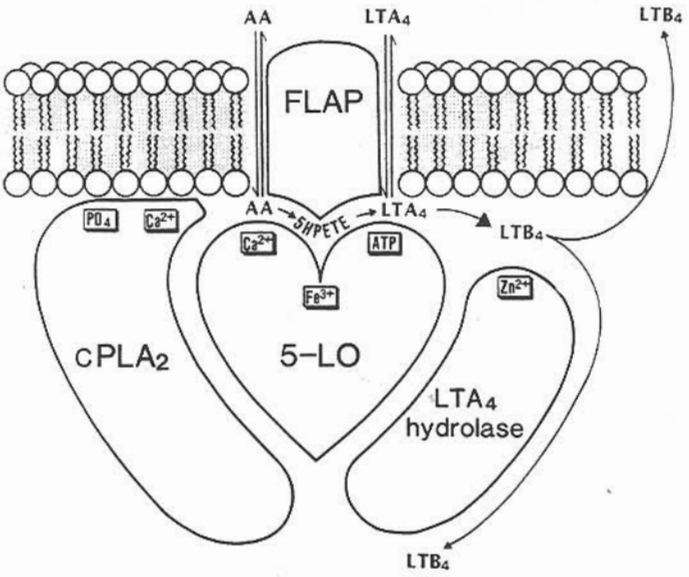

Figure 1. Proposed schematic diagram of the interactions of cytosolic phospholipase A_2 (cPLA$_2$), 5-lipoxygenase (5-LO), 5-lipoxygenase binding protein (FLAP), and LTA$_4$ hydrolase. FLAP is anchored in the perinuclear membrane by its transmembrane domains. The molecular configuration of 5-LO is speculative but derives from the known structure of human serum albumin, which has a heart-shaped cleft for lipids.

mast cells that reside in the ear and skin of the BALB/c mouse differ from the mast cells that reside in the serosal cavity in their expression of mMCP-7 (9) (Table 1) (Figure 3). Ongoing *in vitro* studies suggest that the specific combination of proteases expressed by a mast cell *in vivo* is determined by the cell's cytokine microenvironment. For example, mast cells that are derived by culturing bone marrow cells from the BALB/c mouse in the presence of interleukin (IL)-3 preferentially express mMCP-5, mMCP-6, mMCP-7, and mMC-CPA. However, if these IL-3-developed mast cells are subsequently exposed to the combination of c-*kit* ligand and IL-9, they additionally express mMCP-1, mMCP-2, and mMCP-4 (10). Since the expression of the latter three serine proteases does not occur if IL-4 is added to the media, IL-4 dominantly suppresses the ability of other cytokines to induce protease expression.

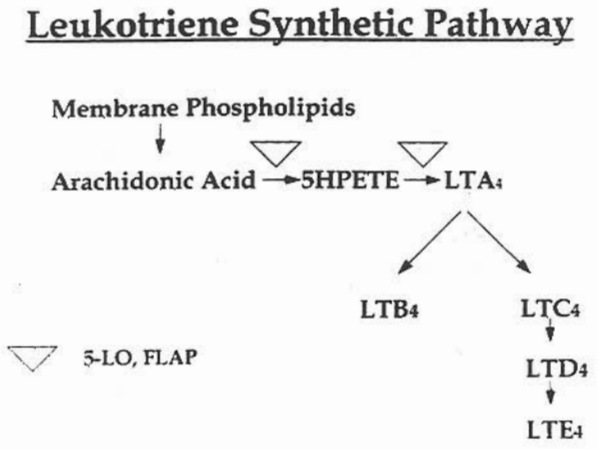

Figure 2. Schema of the metabolic pathways leading to leukotriene formation.

TABLE 1

GRANULE PROTEASES EXPRESSED BY MUCOSAL MAST CELLS (MMC), SEROSAL MAST CELLS (SMC), AND EAR CONNECTIVE TISSUE MAST CELLS (CTMC) OF THE BALB/c MOUSE

Protease Name	Type	MMC[A]	SMC[B]	CTMC[C]
mMCP-1	Chymase	+	–	–
mMCP-2	Chymase	+	–	–
mMCP-3[1]	Chymase	ND	+	ND
mMCP-4[1]	Chymase	–	+	+
mMCP-5	Chymase	–	+	+
mMCP-6	Tryptase	–	+	+
mMCP-7	Tryptase	–	–	+
mMC-CPA	Exopeptidase	–	+	+

Definition of abbreviations: ND = not determined; [A]MMC = mucosal mast cell; [B]SMC = serosal mast cell; [C]CTMC = connective tissue mast cell.

Three-dimensional protein modeling studies (11, 12) have given insight as to why mast cells express so many neutral proteases, and genomic organization studies (13) have given insight as to how their genes evolved. Serine protease genes have been found on almost every mouse chromosome. Surprisingly, the mouse mast cell tryptase (mMCP-6 and mMCP-7) genes were found clustered on chromosome 17, and the mouse mast cell chymase (mMCP-1 to mMCP-5) genes were found clustered on chromosome 14 at the locus that contains the genes that encode cathepsin G and granzymes B, C, E, and F. Whether the clustering of these protease genes influences their ability to be transcribed is under investigation. However, it appears that during the evolution of the mast cell family of chymases, three amino acids were added near the N-terminus and eight amino acids, including a disulfide bond, were deleted near the C-terminus. Protein modeling studies have suggested that these conserved mutations caused conformational changes in the mouse mast cell chymases relative to chymotrypsin, which created potentially new binding sites for substrates. Apparently there was strong evolutionary pressure for a primordial gene containing this structural feature to duplicate at chromosome 14 to give us a number of serine proteases of more restricted substrate specificity than that of pancreatic chymotrypsin so that our immune effector cells could achieve discrete, controlled cleavage of proteins.

One of the unique features of mast cell proteases is that they are stored in the secretory granules in enzymatically active forms. While it has been suspected for some time that serglycin proteoglycans prevent their autolysis in the granule, the mechanism by which this occurs was unknown. Three-dimensional modeling studies (11, 12) suggest that when the mast cell proteases are properly folded, specific basically charged amino acid residues are aligned on the surface of the protease in such a way that they can ionically bind to acidically charged monosaccharides in glycosaminoglycans. In response to a variety of stimuli, mast cells exocytose all of their granule proteases. Nevertheless, some of the exocytosed proteases remain bound to the serglycin proteoglycan as a large macromolecular complex. Analysis of the deduced amino acid sequence of the mast cell proteases suggest that whether the mMCP is a histidine-rich protein or a lysine/arginine-rich protein determines whether it will dissociate from its proteoglycan once exocytosed from the cell.

In summary, substantial progress has been made in characterizing mast cell proteases and their genes. Cytokines have been identified that either stimulate or suppress their expression, and insight has been obtained as to how these proteases are packaged in the secretory granule. Nevertheless, less is known about their cleavage profiles and substrate specificities. The precise role of these proteases in mediating airway pathobiology is therefore an important area for future investigation.

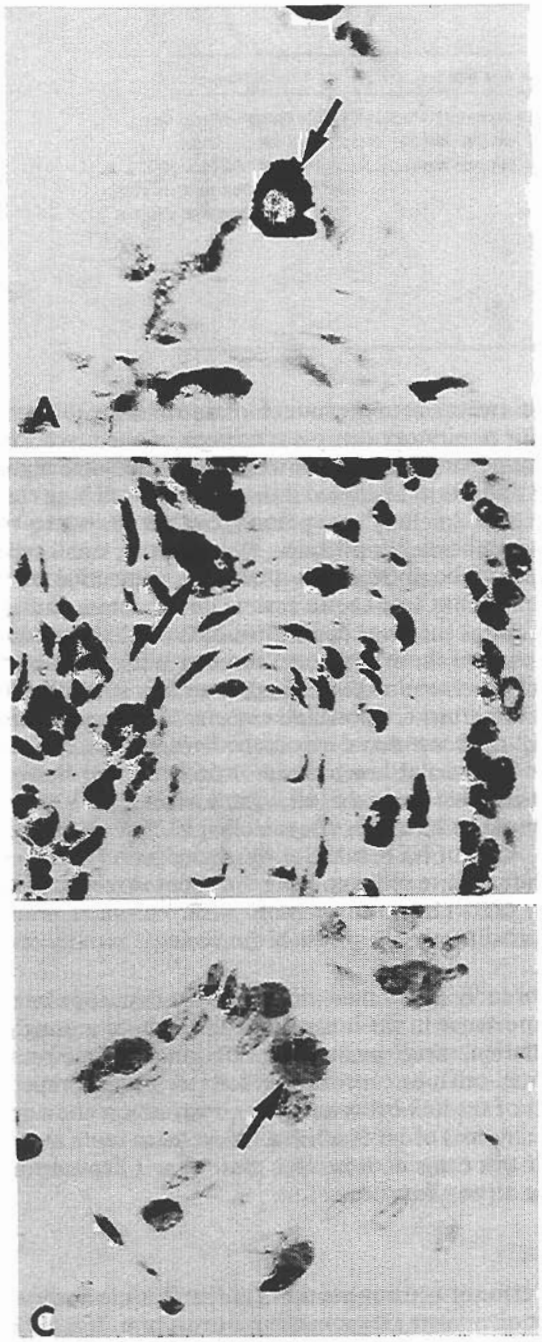

Figure 3. (*A*) Metachromatic staining of mast cell secretory granules in the cytoplasm (arrow) of a Hematoxylin: Acure II: Eosin-stained plastic secretion of mouse ear skin. (*B*) Metachromatic staining of mast cell secretory granules in the cytoplasm (arrow) of a Hematoxylin: Azure II: Eosin-stained plastic secretion of mouse ear skin. Reacted with antipeptide antibodies to the mouse mast cell tryptase (mMCP-7); the red alkaline phosphatase immunocytochemical reaction product (arrow) fills the mast cell cytoplasm. The cells are also positive for mouse mast cell chymases 4–6 and the exopeptidase, carboxypeptidase A. (*C*) The red immunocytochemical reaction product (arrow) delineates mast cells in the gastric mucosa containing the mouse mast cell specific protease 2. Figure supplied and printed with permission from Dr. Daniel Friend.

NEUTRAL ENDOPEPTIDASE

Neutral endopeptidase (NEP) is a membrane-bound ectoenzyme that is an important biological regulator. Understanding this role derives in part from the discovery of its structure. The common acute leukemia antigen (CALLA), or CD10, is an epitope known to be expressed on certain malignant cells. Using a classic approach of protein purification, sequence identification, and library screening, the CD10 epitope was isolated, cloned, and sequenced (14, 15). When the structure was entered into the genbank records, it was found to be virtually identical to that of an enzyme known as neutral endopeptidase 24.11 (NEP) or enkephalinase (15–17) (Figure 4). Indeed, the broad biologic potential of this enzyme derives from its control of the biologic activity of small peptides in the microenvironment of a variety of different cell types (18). Although NEP has not been crystallized, a closely related bacterial protease thermolysin has been crystallized. Like thermolysin, NEP has a His-Glu-X-X-His zinc-binding motif in its active site, hence the designation of NEP as a metalloendopeptidase (Table 2).

It is now well established that NEP peptide substrates include bradykinin, substance P, neurokinin A, vasoactive intestinal peptide, atrial naturetic factor, met- and leu-enkephaline, bombesin, and bombesin-like peptides. The enzyme cleaves these peptides on the amino terminus side of hydrophobic residues; in many of these peptides, cleavage by NEP results in loss of their biologic activities. NEP modulates cellular responses to peptide hormones by regulating local concentrations of peptides at the level of the cell surface receptor (18). Thus, there is reason to believe that NEP competes with peptide receptors for its substrates. Thus, the observed biologic activity of a given molecule reflects com-

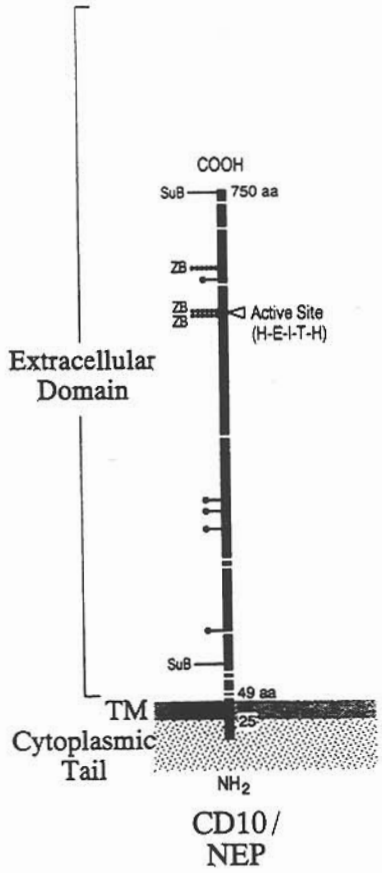

Figure 4. Molecular structure of CD10/NEP. Glycosylation siters are indicated by ●—— and cysteines as a break in the dark line. TM = trans membrane region; SUB = substrate binding site; ZB = zinc binding site.

TABLE 2

	Known Substance	Enzymatic Activity	Cleavage Site	Inhibitors
CD10/NEP	Opioid peptides, fMLP, substance P, bombesin-like peptides, atrial natriuretic factor, endothelin, oxytoxin, bradykinin, angiotensins I and II, vasoactive intestinal peptide.	Cleaves small peptides on the NH_2 terminal side of hydrophobic amino acids.	On amino terminus side of Val, Ile, Phe, Leu, or Ala residues.	Phosphoramindon, thiorphan SCH32615. ACE inhibitors may also inhibit this enzyme.

petition between a given receptor, its ligand, and NEP. It is important to note that peptide receptors are usually on the same cell as neutral endopeptidase, allowing an effective competition between the two entities (Figure 5).

There are a variety of conditions that serve as examples for this competition between NEP and peptide receptors. Importantly, NEP is known to regulate neural inflammation in the lung (19, 20). For example, substance P (SP) and neurokinin A (NKA), which can be released from bronchial C fibers, have the capacity to mediate bronchovascular leak, airway constriction, and mucus secretion. When NEP is present and active, there is a dramatic downregulation of the biologic effects of these peptides such that the SP or NKA released from sensory nerves is inactivated before transducing a biologic effect. However, if NEP is inhibited, using specific enzyme inhibitors such as phosphoramidon or thiorphan, then the biologic activity of these endogenous peptides is remarkably amplified (19, 20). In this regard it is important to understand that NEP activity in the airway can be regulated by a variety of mechanisms, including cigarette smoke, which inactivates the enzyme product, inflammatory stimulation, which can downregulate NEP activity through diminishing NEP message transcription or by causing the internalization of membrane-bound NEP. NEP activity can be enhanced by glucocorticoid stimulation, which increases the NEP message, or by the recruitment of inflammatory cells, such as polymorphonuclear leukocytes that contain NEP. Conditions in which NEP is downregulated are associated with inflammatory conditions in which the biologic activity of peptides such as SP or NKA is amplified.

NEP also appears to be important in the regulation of cell proliferation (21, 22). For example, the enzyme hydrolyzes bombesin-like peptides (BLP) that stimulate the growth of normal bronchial epithelium, including pulmonary neuroendocrine cells. Indeed, it has been shown that by regulating NEP levels in the maturing murine fetal lung, lung growth and differentiation can be modulated (22). This suggests that inhibitors of NEP activity could be useful treatment in premature infants who are thought to be at risk for respiratory distress syndrome of the newborn.

Another pulmonary condition in which NEP-mediated regulation of BLP appears to be altered is that of small-cell lung carcinoma (21). Small-cell lung carcinoma growth is known to be stimulated by bombesin-like peptides. Furthermore, small cells are known to secrete bombesin-like peptides in an autocrine fashion. For example, it has been shown that, in nude mice implanted with small-cell lung cancer cell lines, anti-bombesin-like peptides inhibit the growth of these implanted tumors. It is also known that BLP levels are useful in predicting metastasis in small-cell lung cancer. Furthermore, a potential association between BLP, smoking, and lung cancer may derive from the high levels of BLP found in bronchoalveolar lavage fluid of patients who smoke. Thus, it is possible that the loss of all surface NEP activity might result in enhanced and perhaps uncontrolled BLP-mediated cell growth. An isoform of NEP, in which one exon has been deleted as a result of alternative splicing, has been isolated from a lung cancer library (23). This form of NEP, known as exon 16 del NEP, has approximately 5% of the biologic activity of the native protein.

NEP is a broadly distributed enzyme that appears to have regulatory importance in the lung, particularly in lung growth and differentiation, neurogenic inflammation, asthma, and, potentially, small-cell lung cancer. Indeed, it is tempting to speculate that part of the link between airway obstruction and lung cancer may be the loss of NEP activity. As we learn more about the biology of this enzyme, it is clear that it has a central role in modulating airway function.

SUMMARY

Each of these effector systems has the capacity to initiate airway obstruction or alter airway responsiveness in asthma. It is likely that they act in concert in certain asthmatic settings. Further basic and applied research will define their relative roles in asthma.

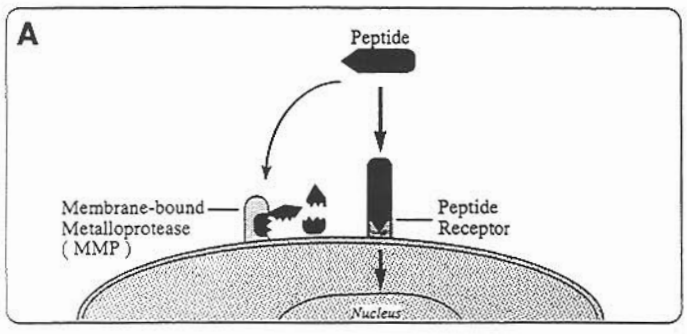

Figure 5. Biologic function of small peptides are regulated by a competition between NEP and the peptide receptor.

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Nonmuscular Airway Obstruction and Asthma

RICHARD C. BOUCHER, ILEEN A. GILBERT, JAMES C. HOGG, MALCOLM KING, MICHAEL R. KNOWLES, and JAY NADEL

It is becoming increasingly clear that the pathophysiology of asthma in the airways involves more than smooth muscle narrowing. A group of four speakers was selected to characterize the nonsmooth muscle factors that contribute to airway wall narrowing and, consequently, airflow obstruction in patients with asthma.

Dr. James Hogg, University of British Columbia, initially described morphometric observations that were interfaced with computer models to predict the consequences of regional differences in airway narrowing as well as the consequences of fixed versus variable obstructions. These techniques will be used to develop more sophisticated morphometric techniques for measuring airway wall thickness with respect to airway wall size in different regions of the lung.

Morphometric analyses of fresh excised lungs from patients with asthma, chronic bronchitis, and a control group revealed that asthmatic lungs are characterized by a uniform thickness of airway walls compared with patients with chronic bronchitis or control subjects. The thickening of the airway wall appears to reflect expansion of all elements in the airway wall, including smooth muscle, the interstitium, and the vasculature. Morphometric data, combined with model analyses, predict that changes in the small airways with regard to airway wall thickness would produce the largest changes in airflow resistance in the lung. Further analysis of the thickening in asthma revealed that the bronchial circulation, both the subepithelial microcirculation and the peri-smooth muscle circulation, was increased in patients with asthma. However, the release in vasculature was proportional to other components of the airway wall and appeared to reflect a general hypertrophy of the interstitium in this disease.

A search was made for etiology of the hypertrophy of airway wall components, i.e., airway wall thickness. A series of immunocytochemical and *in situ* hybridization studies were focused on potential growth factors that may be involved. Whereas immunoreactive TGFβ and PDGFβ were identified in airway walls of patients with asthma or chronic bronchitis and in normal subjects, there appears to be little difference in the intensity of expression or distribution of expression to account for the general airway wall thickening unique to asthma. Thus, it appears that the airway wall thickening is important for airflow obstruction, perhaps most important in small airways, and the etiology will require a general analysis of the consequences of the asthmatic inflammatory process.

Dr. Ileen Gilbert, Case Western Reserve University, focused the discussion on the role of airway wall circulation in the airflow obstruction in asthma. A major difference in the response of asthmatic patients, compared with normal subjects, to the intra-airway cooling that is produced by an exercise on hyperventilation challenge is that asthmatic patients rewarm their airways more rapidly during the immediate recovery phase following the bronchoprovocation (1) (Figure 1). This may reflect increased submucosal blood flow, vascular engorgement, and/or permeability changes that lead to the leakage of fluid from the airway vessels into the surrounding endobronchium. This process itself or in combination with bronchial smooth muscle contraction could produce the mechanical decrements induced by thermal stimuli. The greater intra-airway cooling that occurs during hyperpnea, the more rapid the rewarming in the recovery phase of the challenge and the greater the subsequent airway obstruction. Agents that could potentially mitigate posthyperpnea vasodilation and/or vascular leakage (i.e., topically applied alpha-agonist vasconstrictors, such as norepinephrine) have been shown to decrease the rate and magnitude of posthyperpnea rewarming as well as the obstructive consequences of frigid air hyperpnea (2) (Figure 2).

A number of maneuvers were explored to test the proposed role of the microvasculature in producing airway obstruction in normal subjects and patients with asthma. Airway wall submucosal vascular volume was expanded in both groups of subjects by intravenous infusion of normal saline. When 30 ml/kg of normal saline was rapidly administered, the asthmatic patients developed airway obstruction comparable to a moderately severe thermal challenge (3) (Figure 3). Significantly fewer changes in pulmonary mechanics occurred in the control group, highlighting potential differences in the airway vascular responses of the two groups. In asthmatic patients one third of the volume of infused saline produces similar obstructive responses to the higher volume challenge; consequently, it is unlikely that simple vasodilation or enhancement of blood flow induced the observed mechanical decrements, and the ease with which fluid can extravasate from the microcirculation may be the most pertinent factor.

A role of the bronchial submucosal vasculature in protecting the airway wall from the cooling and drying effects of thermal and hyperosmolar stimuli was also described. Maneuvers that provide an added heat source to the airways to warm and humidify the thermal burden of these challenges before and during their application, i.e., inhaled vasodilators such as furosemide (4) and enalaprilat, shifting blood from the peripheral extremities toward the central circulation by inflation of the lower limb bladders of anti-shock trousers (5), or prior vascular volume expansion with normal saline fluid load (3), mitigate the ultimate degree of airway obstruction produced.

In summary, it appears that the submucosal vasculature plays a major role in airway homeostasis. The microvasculature appears to be able to defend the airway against the effects of surface drying by increasing blood flow to the region. The microvasculature also appears to be a major contributor to rewarming of the airway wall if airway wall temperatures are lowered due to evaporative water loss. Assessing the contribution to airflow resistance of these adaptive maneuvers, which are potentially unique to asthmatic patients, will require analyses of increased blood flow and increased permeability of water into the submucosal compartment, particularly around the smooth muscle.

Dr. Michael Knowles discussed the possible contribution of intralumenal contents to airflow obstruction in asthma. Dr. Knowles reviewed the current states of knowledge and the controversies that surround an integrated description of surface liquid metabolism in the entire pulmonary system. Also reviewed were the vast regional differences in surface area between the alveolar surfaces and proximal airway regions, with a more than 4000-fold reduction in surface area as one moves proximally, and the hypotheses that describe potential sites of surface liquid secretion and absorption, as shown in Figure 4. Best described are the functions of proximal airways epithelium, which appear to absorb liquid at a rate of 5–10 μl/cm²/h, driven in part by active Na^+ transport and passive oncotic and hydrostatic forces. A ma-

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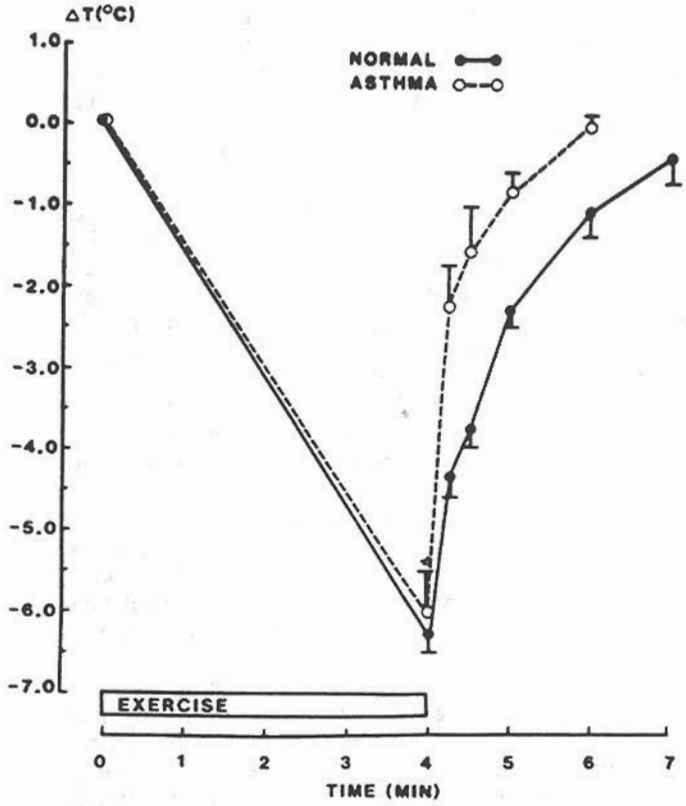

Figure 1. Changes in inspiratory airway temperature (Δt) in trachea during exercise and recovery for normal and asthmatic subjects as a function of time. First two recovery data points are at 15 and 30 s; thereafter, they are shown at minute intervals. Data points and mean value bars, ± SE. Duration of exercise is shown by rectangle at bottom of graph.

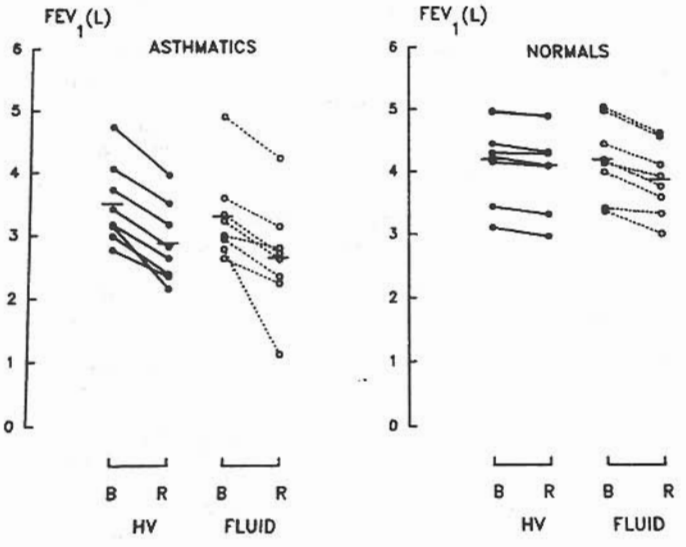

Figure 2. The maximum change in forced expiratory volume in 1 s (FEV_1) before and after isocapnic hyperventilation (HV) (●——●) and saline infusion (FLUID) (○ – – – ○) in the asthmatic and normal subjects. The data points are individual values and the bars indicate the mean; B = baseline; R = maximum response.

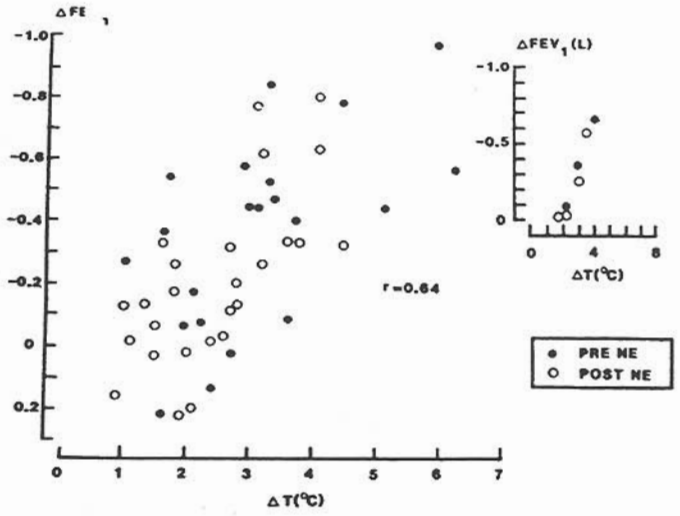

Figure 3. Relationship between the hyperpnea recovery temperature differences at 15 s after hyperpnea (Δt), with and without norepinephrine, and the change in ΔFEV_1. The data points are individual values. The inset shows the relation derived from the mean data.

jor deficit in the analysis is the lack of firm indication of where the lipids that move up the airway surfaces are secreted, i.e., across the small airway surfaces or the alveolar surfaces.

Dr. Knowles then reviewed the effects of pharmacologic agents that perturb the balance of water on airway surfaces on the mucociliary clearance apparatus and lung function. Because the pharmacologic addition of water could be most critical in small airways, the site at which the internal diameter would be most perturbed by small additions of liquid, tests were focused on indices of airflow obstruction of small airways, maximal midexpiratory flow (MMEF), and gas exchange (Po_2, Pco_2). Using a combination of an inhaled Na^+ channel blocker, amiloride, and an agonist that increases liquid secretion, uridine triphosphate (UTP), Dr. Knowles presented data that suggest that MMEF was not perturbed by inhalation of these agents, but small drops in Po_2 could be detected. These effects were more modest than might be expected in the small airways with added liquid, but independent measurements suggested that mucociliary clearance rates were tripled by the addition of UTP, suggesting that the added liquid is cleared axially, perhaps by the effects of UTP on ciliary

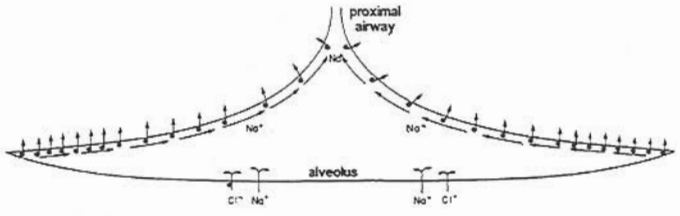

Figure 4. Schematic representation of intraregional surface liquid metabolism. The relative surface areas of the alveolar and airway regions are depicted as a function of the diameters of the two regions. Arrows with dark circles depict active transport, whereas arrows without circles depict passive ion transport. Two possible routes (active Cl^- secretion [*left*] and passive salt and liquid movement [*right*]) are depicted for the alveolar region. In the airways, active Na^+ absorption dominates.

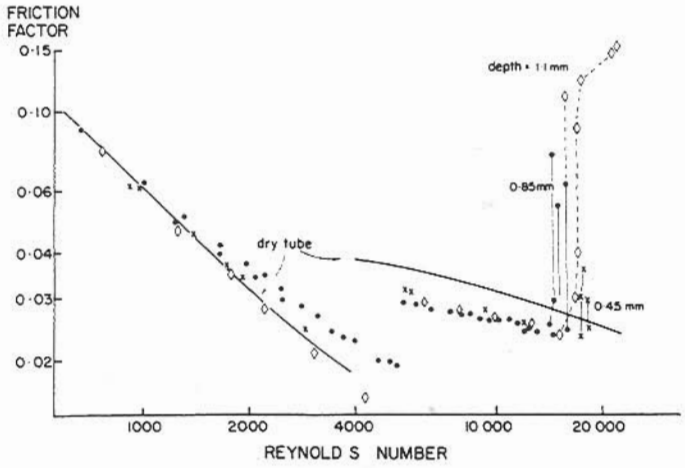

Figure 5. Moody diagram (friction vs. Reynolds number) for air flow through a rigid tube (diameter, 1.85 cm) lined to various depths with a mucus simulant (lightly crosslinked locust bean gum). At moderate flow rates (Re 5,000–15,000), the reduction in friction factor from that expected for simple narrowing indicates damping of airflow disturbances by the viscoelastic lining layer. At high flow rates, the rapid increase in friction is associated with wave formation and axial transfer of lining fluid.

beat frequency. Dr. Knowles concluded with the acknowledgment that little is known about surface liquid volume or ionic composition in asthma.

Dr. Malcolm King, University of Alberta, reviewed the potential contributions of surface liquid mucins to airflow resistance. Discussing a concept reminiscent of the effects of mucin viscoelastic properties on mucin transport rates, Dr. King reviewed data that suggested that the mucins that line airway surfaces have little effect on airflow resistance when air flows at relatively low rates, i.e., in laminar flow, and may even reduce airflow resistance at intermediate flow rates, but at high, turbulent flow rates, they may contribute to increased airway resistance (6) (Figure 5). Dr. King then reviewed data from reports of asthmatic patients in which mucin biorheologic properties have been described as both more or less viscous than normal (7–9). Dr. King echoed a theme that it is difficult to collect mucin from these patients, and there is a paucity of data. He also suggested that, in addition to viscoelastic properties, the adhesiveness of mucin to airway surfaces should be measured in these studies.

Dr. Jay Nadel, Cardiovascular Research Institute, finished the session with a description of the mechanisms that control the amount of mucin secreted onto airway surfaces and its possible contribution to airflow resistance in asthma. Dr. Nadel initially reviewed the data indicating asthmatic patients have more goblet cells in their superficial epithelium than normal, i.e., they exhibit goblet cell hyperplasia, and they appear to exhibit glandular hypertrophy. Dr. Nadel then reviewed the current knowledge base that describes the factors that may control the rate of release of mucins from goblet cells. Borrowing from studies of cystic fibrosis (CF) pathogenesis, Dr. Nadel described a major role for neutrophil-derived proteinases, e.g., elastase and cathepsin G, as mucin secretagogues from goblet cells and gland cells at concentrations starting at 10^{-10} M. Dr. Nadel then reviewed the pathogenesis of the high concentrations of elastase observed in CF secretions, which appears to involve release of a variety of substances by *Pseudomonas aeruginosa*, which stimulate airway epithelium to produce high quantities of IL-8. IL-8 then recruits large numbers of polymorphonuclear cells in the airway lumen that release the secretagogue proteinases.

Dr. Nadel reiterated the difficulties in studying asthma with regard to mucins, i.e., the patients do not expectorate mucin but appear to retain it. A key issue highlighted was whether the abundant mucin in the lung of asthmatic patients observed at autopsy has been there chronically or is explosively released. Further, the role of IL-8, polymorphonuclear cell (PMNs) mucin secretagogues, and potentially the role of infectious agents that could recruit PMNs via an IL-8 mechanism were reviewed.

Finally, Dr. Nadel highlighted the major problem of studying secretions and other phenomena in asthma. In brief, these patients bring up relatively little sputum. Also, unlike CF patients, they do not require transplantation; consequently, it is difficult to obtain tissue. Clearly, based on the model of other diseases, e.g., CF, a complete analysis of expectorated interlumenal secretions and analysis of retained contents in the airway are mandatory to understand the pathogenesis of intralumenal secretions and airflow obstruction.

This session highlighted the potential roles of nonsmooth muscle constrictor functions in airflow obstruction. The key issues that must be addressed are the nature of the stimuli that appear to lead to airway wall thickening that is virtually unique to asthma, potential differences in the regulation of the submucosal microvasculature in asthma, and the composition of airway secretions, i.e., ion, water, and mucin, and a balance sheet of the production and clearance of mucin in the airways of asthmatic patients.

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Approaches to Mapping Genes for Allergy and Asthma

DEBORAH A. MEYERS and EUGENE R. BLEECKER

There is major interest in investigating the genetic components of allergy and asthma. Four different areas are involved in the study of complex genetic diseases. They include family studies, assessment of phenotype, segregation analysis, and gene mapping. Each of these areas is complex and will provide useful information about the natural history and underlying pathophysiologic mechanisms responsible for these disorders. Initial assessment of phenotype must be practical, reproducible, and relatively independent from compounding variables. Possible phenotypes important in allergy and asthma include atopic parameters such as total serum IgE, bronchial hyperresponsiveness, and the presence or absence of clinical asthma. Scientific evidence supporting these studies is found in numerous family and twin studies that have suggested the presence of a heritable component for allergy, bronchial hyperresponsiveness, and asthma. The number of genes involved in these complex genetic disorders and their mode of inheritance has not been fully determined. Initial results of the classification of family members show that approximately 26% of the offspring of families ascertained through a parent with asthma have an asthmatic phenotype. A large number of these offspring with clinical evidence of asthma do not have a prior physician diagnosis of asthma, suggesting that there is a spectrum that ranges from preclinical to symptomatic asthma. The familial aggregation of asthma and other obstructive airway diseases in these families is consistent with a significant genetic component. Linkage studies have been performed on two characteristics of the allergic and asthmatic phenotype. Segregation analysis of total serum IgE provided evidence for a recessive mode of inheritance. Recent linkage data suggest that regions of 5q are important in the regulation of total serum IgE in allergy and asthma. Efforts to more precisely map this region will improve our understanding of the regulation of inflammation in both of these disorders. These genetic studies on allergy and asthma should identify basic immunologic and pathophysiologic mechanisms that are important in these disorders. They may also lead to new specific therapies or methodology for presymptomatic diagnosis and disease prevention.

FAMILY STUDIES AND DEFINITION OF THE ASTHMATIC PHENOTYPE

Families are usually ascertained through a proband, for example, one with classic asthma. Once a family is selected, all family members should be evaluated with a questionnaire (subjective measures) as well as with complete laboratory testing (objective measures). The important concept in family genetic studies is that all of the other family members should be characterized whenever possible. Reliance on historical data alone may result in both false-negative and false-positive diagnoses. For example, an asthmatic proband may have a sibling who displays bronchial hyperresponsiveness (BHR) but may also have a significant smoking history, which will make it difficult to determine the specific trigger that caused bronchial hyperresponsiveness.

To ascertain subjects with an asthmatic phenotype, current approaches for defining asthma should be reviewed. For example, from the national and international guidelines, asthma is defined as a chronic inflammatory disorder of the airways in which numerous cells play an important role, including mast cells, eosinophils, and mononuclear cells (1, 2). Activation of these cells causes release of inflammatory mediators that produce direct effects in the airways as well as triggers chronic cellular inflammation by attracting and activating other cellular elements into the airways. In susceptible individuals this inflammatory cascade causes symptoms that are usually associated with variable airflow obstruction and an increase in bronchial responsiveness. An important practical question is whether this or other definitions help differentiate asthma from other forms of obstructive lung disease and is useful in assessing the presence of preclinical asthma.

If asthma cannot be defined in all family members, an alternative approach is to characterize the phenotype in terms of those parameters that are closely associated with asthma. The assessment of phenotype has to be practical and feasible, reproducible, and relatively independent from confounding variables. The types of confounding variables that need to be considered are age, gender, and environmental exposures. Clearly, when a family is studied, even if environmental influences are assessed at a specific time, changes in that parameter may still need to be studied over time.

Two potential phenotypes are bronchial hyperresponsiveness and total serum IgE, since increased IgE levels reflect allergy, BHR, and even asthma (3–5). These are objective measures that can be evaluated as quantitative tracts and are less subjective than historical or questionnaire data. These measures may be reproducible, but they do vary over time with retesting, possibly related to the effects of age, recent environmental exposures, or the effects of viral respiratory infections (6). The other areas that should be considered are indices of inflammation. Unfortunately, these measures often rely on invasive techniques and cannot be performed in a large number of family members (7). Also, there may be considerable overlap between the allergic phenotype and inflammatory changes found in the airways of cigarette smokers, asthmatics, and allergic asthmatics. Thus, even these invasive approaches may not provide ideal differentiation among subjects with obstructive pulmonary disease.

While there are inherent difficulties involved in the differential diagnosis and characterization of asthma and assignment of a specific phenotype in families of asthmatic probands, these approaches provide useful information that justifies investigation of the genetics of allergic disorders and asthma. Recently, an algorithm has been developed to classify the offspring from families with asthma in northern Holland (8). In the analysis of 230 of the first generation offspring, 18% fell into the "definite asthma" category, and 8 percent into the "probable asthma" category. Thus, approximately a quarter of the children appear to have an asthma phenotype. Another 21% had "unclassified" airway disease (asymptomatic BHR or cigarette smokers with asthmatic characteristics), 4% chronic obstructive pulmonary dis-

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ease (COPD), and 49% normal. Thus, these descriptive clinical data support the role of genetic factors in the development of asthma. It was interesting that approximately 40% of the subjects who appeared to have a definite or probable diagnosis of asthma using questionnaire and laboratory studies did not have a prior physician diagnosis. These findings imply that exposures to immunologic or inflammatory agents may trigger clinical disease in "at risk" individuals who have a hereditary predisposition for the development of asthma.

THE ASSESSMENT OF BRONCHIAL HYPERRESPONSIVENESS IN ASTHMA

It is important to remember that phenotype is predicated on fully understanding the pathophysiology of the disease that is being studied. However, a basic problem with asthma is that we have an imperfect understanding of the pathophysiology of asthma. For example, all subjects with the phenotype of BHR are not symptomatic, which raises the question of the clinical implications of having BHR alone.

Since BHR is a very common finding in asthma that is also frequently associated with allergic status, it is worthwhile to consider some aspects of the assessment and implications of BHR. This trait is almost universally present in subjects with asthma, as well as some allergic individuals and many cigarette smokers with early COPD (9–11). There are also problems in defining the best test to assess BHR.

For example, PD_{20} FEV_1 may be dependent on the baseline levels of pulmonary function. In a child, a fall of 200 ml may be all that is necessary to achieve a 20% decrease in FEV_1, but 200 ml may be within the variance of the test procedure in an adult, where a decrease of 1 L may be necessary for a 20% fall in FEV_1. This raises the question that one potential reason why children may appear to grow out of their asthma or demonstrate less airway hyperresponsiveness is because their lungs grow physically. Another bias that may occur in family studies is the inability to test a family member if their baseline lung function is too low. Obviously, although BHR may be present at birth, there are numerous environmental influences, such as individual and parental smoking, exposures to air pollutants, and viral respiratory infections, that may influence the degree of BHR (6, 10, 11).

Although it is possible to analyze BHR as a continuous measure, total serum IgE levels should also be studied since they explain approximately 30% of the variance in airway reactivity (5). Thus, family studies of both BHR and total serum IgE levels are important in understanding two important parameters that may lead to or even be a risk factor for the subsequent development of asthma.

GENETIC ANALYTICAL APPROACHES AND GENE MAPPING

The same approaches that are used to map genes responsible for Mendelian disorders are used to map genes for complex genetic disorders, e.g., conditions that may be caused by two or more genes and are often influenced by nonhereditary environmental influences (12, 13). However, there are several problems that need to be considered. To take full advantage of the family data, it is best if a genetic model of inheritance can be determined using segregation analysis. Using this method, one can determine the mode of inheritance (recessive, dominant, polygenic, etc.) if the trait or disorder has a major genetic component. This information can be used for linkage studies to calculate LOD scores, a measure of the likelihood or probability that linkage exists between a chromosomal region and a specific disorder. Another approach is to analyze data from affected sib-pairs, which allows one to avoid some of the age-related problems in phenotype determination, such as a significant smoking history in a parent with BHR and respiratory symptoms. With affected sib-pair analysis, it is possible to test if regions of the genome are identical-by-descent, a way to test for possible linkage without knowing the specific mode of inheritance for the disorder (14).

Each approach has advantages and disadvantages, and the investigator needs to carefully consider the aims of their study to decide on their study design. If the families are obtained randomly or by only one affected individual, then segregation analysis can be performed. Otherwise, sib-pair analysis and other nonparametric techniques should be employed initially. Chromosomal markers throughout the genome can be used, and a complete genome search can be performed. The alternative is to study chromosomal regions with candidate genes that may be important in the development of allergy and asthma. A full genome screen can still be performed after investigating potentially important candidate regions.

With an LOD score of 3 (1000:1 odds), there is an error rate of about 5%. This is because it is relatively rare for 2 loci to be linked. If you strongly disbelieve a hypothesis, more evidence may be required. In addition, this assumes that we have a genetic model for gene mapping, which is another problem in a complex disease such as asthma. However, LOD scores in the 2 to 3 range that have been reported in complex diseases have led to finding relevant genes. Therefore, it is difficult to determine the exact LOD score or p value from nonparametric analysis that is needed to declare statistical significance.

It is important to also worry about type 2 errors, the possibility of failing to detect a linkage and missing a gene. It may be cost effective to perform the linkage study in two stages. For the first stage, the markers are sparsely spaced throughout the genome. However, since it is important not to miss a gene that may be present, a lower significance value may be sufficient for preliminary evidence of linkage at the first stage. When any evidence for linkage is found, more markers would be typed in that region. Investigators need to consider the consistency of their results given different analytical approaches and whether relevant candidate genes are in the given chromosomal region. Replication studies are very important to avoid reporting false evidence for linkage.

CURRENT APPROACHES TO GENETIC STUDIES OF ALLERGY AND ASTHMA

Several approaches are being used to evaluate the genetic components of allergy and asthma. While genome searches are planned, most recent data have been obtained studying candidate regions where genes that may be important in these disorders can be found. Cookson and coworkers (15) have reported linkage to an area of chromosome 11q with a broadly defined allergic phenotype. Other investigators have been unable to replicate these linkage results (16–18). However, recently this group has reported a polymorphism within a high affinity IgE receptor that is located in this area of chromosome 11q (19). These investigators have reported that this polymorphism is associated with maternal inheritance of the atopic phenotype in 17% of the alleric families evaluated. Recently, there have been reports of linkage of a locus for total serum IgE to markers on chromosome 5q. This area is rich in a number of proinflammatory regulator cytokines that are potentially important in asthma and allergy. These findings have been reported in two different family studies; one in an inbred population based on ascertainment through an atopic proband (20). The other study was performed in families that were ascertained through a proband with asthma where a recessive mode of inheritance of high IgE levels was found (21). The Figure illustrates the results of sib-pair analysis and LOD score analysis showing linkage to markers on chromosome 5q (21). Initially, several polymorphic markers were genotyped in families identified by having a parent with asthma. Genetic

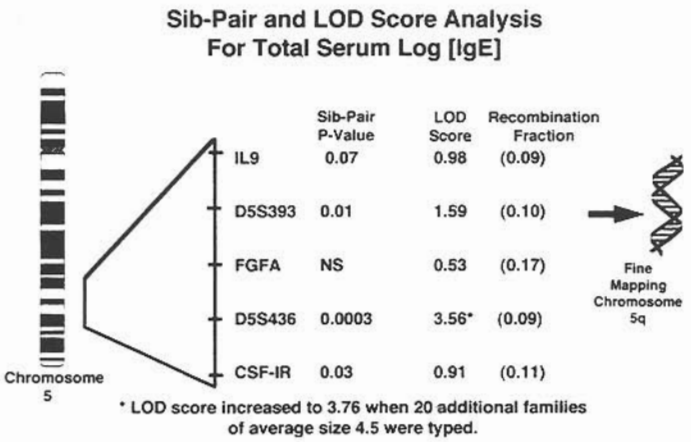

Figure 1. Results of sib-pair and LOD score analysis for total serum IgE in families with asthma. Polymorphic DNA markers on chromosome 5q show linkage to total serum IgE. Fine mapping of this area is necessary to determine the gene(s) responsible for this response (21).

analysis showed significant results for marker D5S436 for sib-pair and LOD score analysis. The LOD value of 3.56 correlates with greater than 1000:1 odds for linkage of IgE to this chromosomal region. IgE appears to be an important determinant of allergy, BHR, and asthma in various population groups. Additional studies are necessary to perform fine mapping of these areas to determine the specific genes responsible for these linkages (Figure).

Genetic studies on allergy and asthma should identify basic immunologic and pathophysiologic mechanisms that are important in these disorders. These approaches may also lead to new specific therapies or methodology for presymptomatic diagnosis and disease prevention.

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