INVESTIGATIONS INTO THE DEVELOPMENT AND MECHANISM OF TOLERANCE INDUCED BY THE CHRONIC ANTIGEN CHALLENGE OF SENSITIZED GUINEA PIGS

by

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ABSTRACT

Previous studies have shown that guinea pigs which have been sensitized to ovalbumin (OA) become unreactive to OA aerosol challenge after multiple nonlethal exposures. The present studies were undertaken to define the mechanism(s) of this nonreactivity, or "tolerance". Tolerance was induced in previously sensitized guinea pigs by challenging with OA aerosol one hour/day, five days/week for six weeks. Reactivity was assessed visually and by lung mechanics. Maximum bronchoconstrictive responses were observed in the first two weeks of aerosol challenge, with responses dropping off steadily thereafter until virtually inapparent after five weeks of chronic challenge. Lung mechanics studies provided an objective means of assessing reactivity. Dynamic compliance (Cdyn) and pulmonary resistance (RL) data confirmed initial strong anaphylactic reactions in the early weeks of chronic challenge which steadily declined over the following five weeks. To investigate the stability of tolerance in the absence of regular aerosol challenge, groups of guinea pigs were withheld from challenge for 4, 6, 8, 10, and 24 day periods. No progressive loss of tolerance occurred when aerosol challenges were withheld for 4 to 24 day periods. In vitro contractile responses of airway smooth muscle from sensitized and tolerant guinea pigs revealed comparable histamine responses in the two groups but a significantly
decreased response to OA from tolerant animals. Total lung histamine content was significantly greater in the tolerant animals (5.74 ± 0.76 μg histamine/g wet lung) than in control (3.05 ± 0.33) or sensitized animals (3.30 ± 0.38). Serum from tolerant animals showed a reduced area of blueing compared with serum from sensitized animals by a six hour passive cutaneous anaphylaxis sensitization. Lymphocytes transferred from tolerant guinea pigs to subsequently sensitized animals did not visibly reduce an initial response to OA aerosol challenge. Antigen-induced histamine release from chopped lung preparations was significantly less in tolerant animals (0.34 ± 0.27 μg histamine released/g tissue, 1.0 ± 0.7 % histamine released) than sensitized animals (1.2 ± 0.3 μg histamine released/g tissue, 5.1 ± 1.4 % histamine released) at a low (1.1 x 10^-9 M) OA concentration. It is concluded that antigen-induced mediator release or antibody synthesis may be inhibited in tolerant animals.
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LIST OF ABBREVIATIONS

Ab--antibody
ACD--acid citrate dextrose
ANOVA--analysis of variance
Br--bronchi
BSA--bovine serum albumin
C--control
cAMP--cyclic adenosine 3', 5'-monophosphate
Cdyn--dynamic compliance
cGMP--cyclic guanosine 3', 5'-monophosphate
CFA--complete Freund's adjuvant
CMI--cell-mediated immunity
ConA--Concanavalin A
ECF-A--eosinophil chemotactic factor of anaphylaxis
H--heavy chain portion of Ig
Ig--immunoglobulin
KLH--keyhole limpet haemocyanin
L--light chain portion of Ig
LT--lower trachea
M--molar (moles/litre)
MIF--migration inhibition factor (of macrophages)
min.--minute
MLC--mixed lymphocyte culture
M. Wt.--molecular weight
OA--ovalbumin
PAF--platelet aggregating factor
Par--parenchyma
PBS--phosphate buffered saline
PCA--passive cutaneous anaphylaxis
PG--prostaglandin
PL--transpulmonary pressure
RL--pulmonary resistance
S--sensitized
sec.--second
SFA--suppressive factor of allergy
SRBC--sheep red blood cell
SRS-A--slow-reacting substance of anaphylaxis
T--tolerant
T.V.--tidal volume
UT--upper trachea
GLOSSARY OF TERMS

Antibody—a protein synthesized in response to antigen and which has the ability to bind to that antigen.

Antigen—a substance which stimulates an immune response when present in an animal.

B-cells—B lymphocytes, bone marrow-derived cells which are plasma cell precursors.

Control—guinea pigs not sensitized, never challenged.

Compliance—elasticity of the lung.

Fc receptor—a receptor present on lymphocytes, eosinophils and mast cells which binds the Fc portion of immunoglobulins.

Immunoglobulin—a glycoprotein consisting of 2 identical heavy (H) and 2 identical light (L) chains which functions as an antibody. Divided into classes A, D, E, G, and M.

In vitro—outside the body.

In vivo—within the body.

Lay-offs— withholding of aerosol challenge.

Pulmonary resistance—resistance of airways to airflow.

Sensitized—guinea pigs immunized two weeks previously, never challenged.

Suppressor T-cells—a subset of T lymphocytes which inhibits other effector T-cell immune reactions and B-cell antibody synthesis.

T-cells—T lymphocytes, thymus-derived cells, involved in cell-mediated immune reactions.

Tolerant—guinea pigs immunized, and challenged until non-reactive.
Asthma is a condition which affects approximately 3% of the North American population (1), but is most prevalent as a chronic condition in children. Complete understanding of the cause or causes of asthma and the way in which these alter structure and function is lacking, so a strict etiological definition of asthma is not possible. Individuals have different stimuli which trigger their asthmatic episodes. The stimulus of asthma may be an allergen, exercise, infection, a chemical compound, or a psychogenic event. Whatever the initiation, it culminates in respiratory distress of varying intensity. This variability in dyspnea is the basis for the functional definition of asthma (2). This disorder of function results from variations in resistance to gas flow in narrowed peripheral airways. Spontaneous variability in dyspnea with intervals of freedom from dyspnea and significant decrease of dyspnea by bronchodilators or corticosteroids are important features in defining asthma.

Anatomically, there are characteristic pathologic changes in the airways of asthmatics (3, 4). Again, these are of varying degrees of severity depending upon the patient. Bronchial plugs composed of mucus, serous and cellular elements are found. Hyperplasia and hypertrophy of submucosal glands and goblet cells results in increased mucus secretion. Submucosal inflammation with dilated capillaries contribute to interstitial oedema and the serous component of bronchial plugs. Eosinophilic infiltrates are commonly found in asthma, as is
sloughing of the mucosal layer. These bronchial epithelial cells and eosinophils comprise the cellular portion of the bronchial plugs. Hyperplasia and hypertrophy of bronchial smooth muscle and increased fibrillar collagen adjacent to the basement membrane are further morphologic abnormalities.

Theories of the pathogenesis of asthma are numerous, and only those relevant to allergic asthma will be discussed (3, 4, 5). Whatever the other contributing factors, it is now agreed that the primary event is exposure of the individual to an antigen which is processed by the immune system as an allergen. This induces the production of IgE antibodies which are specific for the allergen, and which bind to mast cells in the interstitium and airway lumen of the lungs. Upon subsequent exposure to the allergen, binding of the allergen to the mast cell-bound IgE causes degranulation of the mast cell with release of its chemical mediators. Some of these mediators are histamine, slow-reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor (ECF-A), platelet aggregating factor (PAF) and prostaglandins (PG). The total effects of these mediators are not completely understood, but the immediate effects appear to be stimulation of irritant receptors leading to rapid shallow breathing via a vagus nerve reflex (6). Increased epithelial permeability by loosening of tight junctions (7) allows the entry of antigen and mediators to the submucosal region, where greater numbers of mast cells are present. Interaction of antigen with IgE bound to interstitial mast cells provokes release of larger quantities of mediators. These
mediators can readily interact with smooth muscle cells, nerve endings and capillaries, producing direct and indirect bronchoconstriction and interstitial oedema and inflammation from leaky capillaries.

Various hypotheses of the pathogenesis of allergic asthma have been investigated in studies in animals and man. It has been found that the airways of asthmatics are extremely sensitive to physical, chemical, and pharmacologic stimuli (4, 8). The abnormality which gives rise to this bronchial hyperreactivity is still uncertain, although several theories are being investigated. There is support for a number of hypotheses, but none of them alone appears to account for all cases. Since these provoking stimuli are antigen-independent a non-immunologic mechanism can also be responsible for bronchoconstriction.

Some possible causes of bronchial hyperreactivity are abnormal parasympathetic, sympathetic or non-adrenergic innervation, increased epithelial permeability, increased smooth muscle responsiveness, or chronically decreased baseline airway calibre.

The main nervous control of airway smooth muscle depends upon a balance between the bronchoconstrictive sympathetic (alpha-adrenergic) and parasympathetic vagal activity and the bronchodilative sympathetic beta-adrenergic and non-adrenergic inhibitory activities (9). Evidence is available that vagal efferent activity produces a mild degree of resting tone in airway smooth muscle (8). Studies in dogs (10) and guinea pigs (6) have shown that in response to antigen or histamine the vagus mediates a reflex bronchoconstriction. Experiments with bovine
lungs have shown that tracheal muscle muscarinic binding sites are 37-fold greater than in the lung periphery, while beta-receptors are 8-fold greater in the periphery than in the tracheal muscle (11). Vagal stimulation could thus be expected to produce a marked reduction in the tracheal airway diameter, and although this has been reported by Jackson et al (12) a preferential decrease in tracheal cross-section was not observed, with distal airway cross-sections also being reduced. Woolcock et al (13) have reported differences in central and peripheral resistance in dogs receiving vagal stimulation. An absolute increase in central and peripheral resistance upon stimulation occurred in all dogs. However, individual differences between dogs were observed. In some, the increase in central resistance was greater than the increase in peripheral resistance, and in others, the reverse was observed. Consideration of various possibilities to account for the phenomenon suggested that the most likely explanation was that the distribution of vagal fibres along the tracheobronchial tree varies among dogs. Irritant receptors lying in the bronchial epithelium appear sensitive to these vagal influences and other stimuli (6, 8, 10). It has been suggested (14) that increased vagal nervous activity is responsible for increased airway smooth muscle tone in the asthmatic state.

Other possible defects in airways innervation are in the bronchodilation mechanisms. The sympathetic beta-adrenergic system has been observed in animals (8, 15-19) although no definite contribution in humans has been established (8, 20). A study of guinea pig
experimental asthma (16) has found that the alpha-adrenergic receptors are significantly increased in chronically antigen challenged animals. This is supportive of a theory put forward by Szentivanyi (21) suggesting that increased alpha-adrenergic activity combined with partial beta-adrenergic blockade is a cause of some forms of asthma. Human studies (4, 8) have also provided support for this theory. A non-adrenergic inhibitory system has been well documented in animals (8, 22-24) and humans (8, 18, 20). Vasoactive Intestinal Peptide has been cited as a possible transmitter of non-adrenergic relaxation in guinea pig airways (24), and this non-adrenergic inhibitory innervation has been found to run in the vagi and recurrent laryngeal nerves (22). It has been hypothesized that a defect in this non-adrenergic inhibitory system would result in excessive bronchoconstriction leading to bronchial hyperreactivity (8, 20).

Although evidence has been presented for abnormal function of autonomic regulation in bronchial hyperreactivity, other factors may be involved which are primary or promoting for this defect. One of these may be epithelial damage resulting in increased permeability of the mucosal barrier. The irritant receptors and nerve endings present within the epithelium would then be exposed to stimuli which may produce hyperreactivity either through repeated stimulation or through a "sensitization" of the nerve endings (3, 4, 8). Studies have shown that when challenged with bronchoconstrictors subjects with upper respiratory tract infections had increased bronchial reactivity when compared with normal controls (25).
Airway epithelial damage is an abnormality during respiratory viral infections, and respiratory infection is one of the stimuli of asthma. That epithelial damage is present in asthma and may contribute to hyperreactive airways is conceivable in view of the fact that damaged mucosa is a normal pathological finding in asthma (3). Eosinophilia is also a common finding in asthma and a recent study by Frigas and others (26) demonstrates that the major basic protein which is a component of eosinophils is severely cytotoxic to bronchial epithelium causing ciliary damage, oedema, and disruption of the epithelium. This may be a major contributor to the damage observed in asthmatic bronchial epithelium. Fc receptors for IgE have been detected on human eosinophils, and the number of receptors were found to increase when the eosinophils were preincubated with ECF-A (27). This situation may occur in an allergic individual, but the implications of eosinophil IgE-antigen binding are unclear. Another contribution to airway epithelial damage may be made by macrophages. They have been shown to bind IgE and may play a role in the pathology of asthma by inducing tissue damage (28). The anaphylatoxins C5a and C3a are also capable of inducing contractions of lung airways and parenchyma, and may well be present in the allergic reaction (29).

Another contribution to bronchial hyperreactivity may be made by alterations in the smooth muscle. It is well established that the quantity of smooth muscle is increased in asthma, and an increased amount of muscle is likely to cause a larger narrowing of airways (8). However, this mechanism is unlikely to be totally responsible for
bronchial hyperreactivity since the mass of airway smooth muscle in humans during respiratory infections or exposure to chemical stimuli would not be increased. Another change in the smooth muscle may be a change in its intrinsic contractile properties in response to ions or agonists. The mechanism of this may reside in the sensitivity of smooth muscle to calcium ions. Martorana (30) has shown that guinea pigs sensitized to ovalbumin have calcium ion-binding characteristics which are markedly different from normal. The airway hyperreactivity seen post-anaphylactically may then be due to an increased sensitivity of the smooth muscle cells to extracellular calcium ions (31). Tracheal smooth muscle preparations from ovalbumin-allergic dogs have been shown to be hypersensitive and hyperreactive in response to histamine when compared with littermate controls (32). In contrast to this study on dogs, Souhrada did not find that the reactivity of tracheal smooth muscle from experimental asthmatic guinea pigs was significantly different from that of control tracheal preparations (33). However, Souhrada has demonstrated with sensitized guinea pig smooth muscle in vitro that spontaneous mechanical activity is increased (34) and suggests that this is due to an increased resting membrane potential of the smooth muscle cells (35). The extent of the contribution of altered airway smooth muscle properties on bronchial hyperreactivity remains uncertain.

The presence of mucosal oedema, smooth muscle hyperplasia and hypertrophy, and mucosal folding may all contribute to a narrowing of the baseline airway calibre. Airway flow resistance is a common in
vivo parameter for measuring bronchoconstriction, and resistance is inversely proportional to the fourth power of the radius when airway flow is laminar. Therefore, when already narrow airways are further decreased by bronchoconstriction there is a greater change in airway resistance than there would be if wider airways contracted the same amount. Benson (36) has suggested that narrowing of airways is responsible for bronchial hyperreactivity. However, bronchial hyperreactivity is observed even when narrowing of the baseline airway calibre is absent (7). Although it may contribute to hyperreactivity when it is present, it is clearly not a primary mechanism.

Impaired mucociliary function is also involved in asthma. Some characteristics of this have already been described, such as hypertrophy and hyperplasia of submucosal glands and goblet cells and increased mucus in the airways. There are a number of factors which may contribute to abnormal handling of respiratory secretions. One of these is the aforementioned fact that the epithelial layer is often damaged. Cilia which are absent or have uncoordinated or reduced beating patterns would be less able to clear mucus from the airways. There is also evidence that the mucus in asthmatics is biochemically abnormal (37). Increased concentrations of unusual polysaccharides, serum proteins which cross bind, and secretory IgA have been found in the sputum of asthmatic patients. Increased calcium ions and serum proteins have also been reported in the sol phase of sputum. The greater viscosity of sputum which has been observed in asthmatics may well be due to its altered composition, especially the increased
glycoproteins. It has been found that when viscosity is increased, the rate of transport on ciliated epithelia is decreased. Increased mucus volume and viscosity, and decreased transport provide the conditions for mucus stasis. The fibrin content of mucus increases, and appears as lamina when mucus stagnates in the airways. This too, increases the viscosity of the secretions. Secretion from submucosal glands is regulated at least in part by parasympathetic and sympathetic innervation (38). In vitro studies with cat, dog, ferret, and human trachea have shown that vagus nerve stimulation and alpha-adrenergic agonists stimulate secretion from submucosal glands. If increased activity of the vagus nerve and the alpha-adrenergic system is a part of the pathogenesis of asthma, this may account for the increased mucus secretion. When the ciliary function is impaired, this excess mucus cannot be cleared, and mucus plugs form in the narrowed airways.

Although the studies investigating airway hyperreactivity and mucociliary dysfunction have contributed much to our knowledge of these components of the pathology of bronchial asthma, there is still uncertainty about the basic mechanism(s) of asthma.

Additional experiments have been performed using animal models to investigate the contributions of the immune system, functions of cells and mediators, and the pulmonary pathophysiology of the disease. Some general animal models of the asthmatic state have been described by Patterson and Kelly (39).

As previously stated, the handling of an antigen by the immune system to produce an antibody which will bind to mast cells is an
essential part of allergic asthma. Whether or not the production of these antibodies is the main pathogenesis of asthma is uncertain. The components of the immune system which are thought to be involved in the production of reaginic antibodies will now be discussed.

The first consideration is the route by which the antigen is introduced into the body. Entry via the mucosal surfaces of the respiratory tract or the intestinal tract will allow "sensitization" to the antigen to occur. Evidence has been derived from studies in guinea pigs and mice that indicate that alveolar and peritoneal macrophages are involved in antigen processing and presentation to T-lymphocytes (40-44). Antigen entering via the respiratory tract may be processed by alveolar macrophages, or it may be processed by gut-associated lymphoid tissue. A study by Willoughby (45) has shown that rabbits exposed to BSA aerosol for 90 min. have 72% of the antigen in the stomach immediately after the aerosol exposure. Within the intestinal tract there are two routes by which antigens may be absorbed. One is via the epithelium overlying the lymph nodules, and the other is via the columnar epithelium covering the villi (46). Horseradish peroxidase placed in the lumen of a mouse intestine can be observed in vesicles passing through the "membranous" epithelial cells which separate lymphocytes from gut lumen. Later the horseradish peroxidase is detected in the space between the epithelial cells and lymphocytes, and finally within lymphocytic vesicles. Antigen absorption across columnar epithelium may also occur. Mice and rats given radio-labelled proteins intragastrically have been shown to absorb varying amounts of
the protein in its native state. Distribution of antigens to lymphoid
tissue throughout the body may then occur. Interactions between
macrophages, suppressor and helper T-cells and B-cells are required for
the ultimate formation of antibodies. Some evidence is available which
suggests that a deficiency of suppressor T-cells allows hyperproduction
of IgE, and a resulting atopic state (47).

The production of antibodies is the result of an intricate
shuffling and joining of segments of DNA and RNA within developing B
lymphocytes (48, 49). An antibody (Ab) molecule is composed of two
identical heavy (H) and two identical light (L) polypeptide chains,
each consisting of sequences of polypeptides producing regions termed
"variable" and "constant". There are two types of L chains, kappa (κ)
and lambda (λ), and five classes of H chains, alpha (α), epsilon (ε),
delta (δ), gamma (γ) and mu (μ). Within each type of L or H chain the
constant region of the polypeptide varies little from Ab to Ab.
However, the variable regions vary greatly, and confer
antigen-specificity upon the Ab. In the range of \(10^6-10^8\) unique
antibodies may be produced by an animal. The usual practice of one
gene giving rise to one molecule does not operate in this situation.
Rather, heterogeneity is produced by the random selection of one
variable sequence from among a large number of possible genes.
Although the exact number is not yet known, there is probably in the
range of one hundred to two hundred variable gene sequences for H chain
synthesis and one hundred to two hundred variable gene sequences for L
chain synthesis. During development of a B-cell, the 'chosen' variable
gene is linked to the one constant gene present in the genome. Further variability is introduced by the choice of one of several J genes, a segment which joins the variable and constant regions in L and H chains. Heavy chains have another component of variability, the D or diversity genes, which produce one of the three hypervariable regions of the heavy chain. In addition to the choice of different variable, J and D genes, recombination is flexible over a range of several nucleotides for joining variable and D segments and D and J segments. Further variability is available from H and L chain pairing. Another mechanism for promoting Ab diversity is that of single nucleotide mutations during the course of B lymphocyte maturation.

The arrangement of genes along the DNA molecule is such that the variable genes are followed by the D, J and constant genes. The genes which code for the constant regions of each of the classes of heavy chains are arranged in the order mu, delta, gamma, epsilon and alpha along the DNA molecule. Hence, when transcription occurs, mu and delta are the first genes transcribed and translated. Combination with subsequently synthesized light chain molecules forms complete IgM and IgD molecules. The initial transcription of these genes includes information which provides a hydrophobic end to these molecules, thus ensuring that the first IgM and IgD will be membrane-bound. By this stage, then, rearrangement and hypermutation of multiple germline genes in the developing B lymphocyte have given rise to a B lymphocyte with membrane-bound IgM and IgD, both having the same variable regions and so therefore specific for the same antigen.
Upon binding of the specific antigen, the B lymphocyte is stimulated to continue its development to a fully Ab-producing plasma cell. The membrane-bound Ab are lost, and IgM, IgG, IgE or IgA may be synthesized and secreted. This constitutes a 'heavy chain class switch' and may be accomplished by several mechanisms. One of these is rearrangement of the DNA sequences by means of a similar 'switching signal' between the constant genes and the variable region. The transcribed RNA is then spliced to make a mRNA encoding a gamma, epsilon, or alpha region. Another possibility is that there are class-specific 'switching proteins' (50) which recognize the specific 'switching signal' areas and bind to them, thereby juxtaposing the appropriate regions and allowing DNA recombination.

From this series of events each class of Ab may be synthesized by its own clone of plasma cells which develops from the initial B lymphocyte. IgM and IgG which circulate in blood plasma, IgA which associates with surface-lining cells, and IgE, which binds to allergenic cells may all be produced, undoubtedly subject to control by other modifying factors.

Humans have been found to synthesize a short-term sensitizing antibody (IgGS-TS) in addition to the commonly described IgE (51). Numerous animal models have been used to study the effects of sensitization on antibody levels. Guinea pigs, a commonly used animal model, have been shown to synthesize IgG$_{1a}$, a rapidly sensitizing (4-6 hours), short-lived (3-8 days), heat-stable antibody (52, 53). Other studies have detected the presence of IgE in addition to IgG$_{1}$.
IgE is heat-labile, has a long sensitizing time (48 hours or longer), and persists at least 2-4 weeks in the skin. Rats sensitized with egg albumin and Bordetella pertussis (58, 59) have been shown to produce IgE specific for egg albumin. The assessment of antibody levels is most often done by passive cutaneous anaphylaxis (PCA) experiments (52-58, 60, 61). This involves injecting serum from a sensitized animal intracutaneously into the back of a non-sensitized animal. Antigen and an indicator dye are later injected intravenously. At the sites of serum injection a reaction is seen by a patch of dye, the size of which is proportional to the amount of antibody in the serum from the sensitized animal. In vitro precipitin tests are another means of determining antibody levels (53, 60, 61). IgE can also be measured by a radioimmunosorbent method. This allows measurement of nanogram levels of IgE and enables detection of small differences in total versus specific IgE antibody levels (53, 62).

Much information is now available on the biochemical changes which precede degranulation of mast cells. Degranulation is commonly assessed via histamine release. The first requirement of degranulation is for cross-linking of the IgE receptors in the cell membrane (63-71). These receptors are composed of a 50,000 M. Wt. glycoprotein alpha-component and a 30,000 M. Wt. beta-component in a 1:1 ratio (72, 73). Divalent antigen performs this role in vivo, but anti-IgE antibody, chemically dimerized IgE, anti-receptor antibody, or Concanavalin A (cross-links IgE by binding to Fc region) have been successfully used in vitro. Cross-linking of the IgE receptors leads
to activation of a methyltransferase and adenylate cyclase. An early rise in cAMP is produced which in turn activates two protein kinase isoenzymes (69) the function of which is uncertain. Other biochemical steps include decarboxylation of phosphatidyl serine to form phosphatidyl ethanolamine which is methylated by a magnesium-dependent methyltransferase to produce an N-monomethyl derivative. Phosphatidyl choline is then produced by the action of a second methyltransferase on phosphatidyl ethanolamine. Phosphatidyl choline is a substrate for membrane phospholipase A. The resulting lysophosphatidyl choline appears responsible for opening channels for calcium influx. Increased intracellular calcium is a requirement for degranulation. The production of ATP is also a requirement as is an adequate endogenous level of ascorbic acid (74). The relationship between histamine release and cAMP, cGMP, and PGE\textsubscript{2} levels has been studied by a number of investigators. Conclusive answers are not yet available. The observation was made that during the anaphylactic release of histamine intracellular cAMP levels fell, while cGMP levels rose, producing a decrease in the cAMP/cGMP ratio. The effects of these two cyclic nucleotides thus appears to be opposite, and is part of the "Yin-Yang" theory proposed by Goldberg (75). It has been theorized that a fall in intracellular cAMP is an initiating event for histamine release, since cAMP levels decrease during histamine release, and maintenance of high cAMP prevents the release of histamine (63, 64) by preventing the opening of calcium channels. The inactivation of calcium channels may then occur as cAMP levels increase. However, it
has recently been reported that a short-lived increase in cAMP precedes the fall in cAMP (64, 69, 76). A study using antigen-challenged guinea pig lung slices, minced lung, and perfused lung has shown that the cAMP and cGMP levels change in parallel (76). Both nucleotides exhibited an initial increase, followed by a decrease, then a greater and more prolonged increase. The basic event, however, does not seem to be changes in nucleotide levels. Rat studies by Okabe and others (77) have shown that a fall in PGE$_2$ precedes the decrease in cAMP/cGMP ratio. Infusion of PGE$_2$ elevated the cAMP level, the cAMP/cGMP ratio, and inhibited the antigen-induced elevation of plasma histamine. The finding that prostaglandin (PG) infusion prevents release of histamine from the lung has been reported by others (78, 79). Whatever the precise biochemical sequence of events which result in degranulation, activation of the cytoskeletal elements of the cell (71), possibly via contraction of microfilaments (63), is a necessary step in granule release.

Although histamine has been most often cited as the mediator released by mast cell degranulation, it is by no means the only one present. A variety of mediators, some stored pre-formed, and some synthesized de novo in response to stimulation are released upon degranulation. Functions of these mediators range from bronchospastic and vasoactive activity to chemotactic, enzymatic, and structural activities (71).

Histamine is stored pre-formed and when released acts rapidly upon $H_1$ receptors in smooth muscle of large and small airways to give
a short-lasting bronchoconstriction. Stimulation of irritant receptors causes a reflex neural bronchoconstriction (71, 80). Partial disconnection of vascular endothelium produces vasodilation and increased vascular permeability leading to oedema and inflammation. The receptor on which histamine acts has been reported as $H_1$ (71), $H_2$ (80), and $H_1$ and $H_2$ (81). The action of histamine at an $H_1$ receptor produces an increase in cellular cGMP, while action at an $H_2$ receptor produces increased cAMP. This latter action decreases IgE-mediated histamine release (71) and mediates relaxation (82-85). Histamine has also been shown to relax trachea by stimulation of adrenergic receptors (63). Prostaglandin increases are observed after histamine stimulation, but it is unknown whether this is a direct action of histamine, or a reflex from bronchoconstriction (71, 80).

Histamine causes a release of catecholamines by direct action on the adrenal medulla (80, 86-88). The epinephrine released influences the recovery from histamine, apparently by the beta-effects of the epinephrine (87). In addition, non-adrenal adrenergic factors have been shown to modify responses to histamine (68). The effect of histamine is short-lived. It appears within 30 sec. of antigen challenge, and has reached its maximum within 2 min. (80). The inactivation of histamine occurs by oxidative deamination from histaminase, or methylation plus oxidative deamination from histaminase plus histamine N-methyltransferase (89).

Slow-reacting substance of anaphylaxis (SRS-A), now frequently referred to as the leukotrienes C and D have been assigned a major role
in guinea pig lung anaphylaxis by studies employing the SRS-A receptor antagonist, FPL 55712 (90-92). Upon stimulation of the mast cell, SRS-A is synthesized de novo. It appears within 30 sec. of antigen challenge, but only at a low concentration. Release of SRS-A can still be detected 30 min. after challenge (71, 80) and its release may be inhibited by leukotriene D₄ (93). The type of bronchoconstriction it induces is slow, long-lasting, and contributes to the later, prolonged airway antigen response (94, 95). Vasodilation is also observed with SRS-A stimulation (71), as well as prostaglandin release (71, 80). SRS-A is acid labile, alkaline stable, and stable to heating at 60°C for 60 min. (96). It is formed from arachidonic acid via the lipoxygenase pathway (97, 98). The complete structure of the SRS-A released from an immunologically challenged guinea pig has been determined. It is an unsaturated sulphur-containing lipid of 380-400 M. Wt. (96, 97) which appears to have the same properties from man, guinea pig, and rat (99). The smooth muscle contractions induced by SRS-A require an extracellular source of calcium, and appear to have a similar contraction mechanism to that of histamine and acetylcholine, although specific SRS-A receptors are present on the smooth muscle (100). In guinea pigs the action of SRS-A on peripheral tissues has been found to be significantly greater than the effect on central airways (71, 80, 101), but in humans central and peripheral airways appear to respond to SRS-A to the same degree (102). SRS-A doesn't have a powerful direct contractile effect on the lung, but may indirectly produce bronchoconstriction. Although SRS-A is most often
considered in terms of its release from antigen-stimulated mast cells, other cells appear to produce SRS-A, and may be required for mast cell SRS-A production (71, 103, 104). Furthermore, SRS-A may be released by non-immunological stimuli from other tissues, suggesting a broader physiological role than just anaphylaxis (77). The inactivation of SRS-A is accomplished by arylsulphatases A and B which are stored pre-formed in mast cells (71).

Prostaglandins, a product of arachidonic acid oxidation (71) are also produced upon anaphylaxis. PGE$_2$ and PGF$_{2\alpha}$ are the major PG's which are produced in the lung (80, 105, 106). These PG's have opposing actions, and are produced preferentially in different parts of the lung. PGE$_2$ and its 15-keto metabolite relaxes bronchial smooth muscle and is a vasodilator in a variety of vascular beds (80, 107). PGE$_2$ is produced preferentially in the airways in humans, and is also produced by guinea pig airways. Beta-adrenoceptor activation has been shown to induce PGE$_2$ generation in guinea pig trachea (108). PGF$_{2\alpha}$ and its 15-keto metabolite contract bronchial smooth muscle and are produced primarily by parenchyma (80, 105, 106). The stimulus, source and function of PG production is as yet uncertain. The release of PG's is unrelated to the antigen dose, and it has been variously hypothesized that histamine (H$_1$ stimulation), bronchoconstriction, or tissue damage are the initiating factors for PG production (80, 105, 106). Parenchymal production of PGF$_{2\alpha}$ does not appear to be dependent upon histamine or bronchoconstrictive stimuli (105). The major source of PGF$_{2\alpha}$ in parenchyma may not be the mast cell, but
perhaps another cell type more common in parenchyma than in bronchi (105). Prostaglandins do not appear to make a significant direct contribution to the bronchoconstriction aspect of anaphylaxis, although it has been suggested that the metabolites of PG's may sensitize bronchial smooth muscle to the action of other mediators of anaphylaxis (80). Alternatively, PG's may be involved in a feed-back mechanism whereby histamine, SRS-A and kinins stimulate PG release, then PG's inhibit further mediator release. PGE and PGF$_{2\alpha}$ have been shown to increase cAMP levels in rat peritoneal mast cells and human lung fragments. Via their differential release, PGE$_2$ and PGF$_{2\alpha}$ may maintain ventilation/perfusion ratios in the lung (80). The metabolization of prostaglandins released during anaphylaxis occurs before the PG's reach the pulmonary circulation. The rate-limiting enzyme is 15-OH PG dehydrogenase (80).

Kininogen levels have been observed to decrease during anaphylaxis, thus suggesting that bradykinin is released. Induction of tachyphylaxis to bradykinin produces a decreased bronchoconstriction, thus providing further evidence for the release of bradykinin during anaphylaxis. The smaller airways appear to be selectively narrowed by bradykinin, causing a decrease in lung compliance (80). Another effect is vasodilation, which results in a drop in the systemic blood pressure. However, the release of catecholamines rapidly reverses the initial hypotension. Again, kinins may be required for the release of prostaglandins or other mediators. Peptidases in the blood metabolize the kinins, probably mostly in the pulmonary circulation (80).
A mediator chemotactic for eosinophils, ECF-A, is stored pre-formed in mast cells and is released dose-dependently in a time course similar to that of SRS-A. Its release is modulated by cAMP and cGMP in the same way as histamine and SRS-A. The 400 M. Wt., acidic dipeptide structure attracts eosinophils, and deactivates them to further migration. Eosinophils contain arylsulphatase, which inactivates SRS-A. This suggests the presence of a feed-back mechanism to limit the effect of SRS-A (71, 80).

Another mast cell mediator which acts on another cell type is platelet aggregating factor (PAF). The lipid-like PAF has been shown to promote relaxation of guinea pig trachea (109) as well as inducing the release of serotonin from platelets. This monoamine is stored pre-formed in platelets and has been shown to contract smooth muscle in some non-human systems. Serotonin also increases vascular permeability. Heparin, a structural proteoglycan, is stored pre-formed in mast cells. It functions as an anti-coagulant by interacting with human anti-thrombin III. A variety of enzymes are also present in mast cells, some of which appear to diminish the effects of other anaphylactic mediators (71). Chymase, stored pre-formed, is a protease with alpha-chymotrypsin-like activity, and has been shown to inhibit IgE-dependent inflammation in rabbit skin. Arylsulphatases A and B hydrolyze SRS-A and are released from pre-formed stores. Although there are other mediators and factors present in mast cells, those whose functions are best understood have been described here. The summation of the actions of the mediators of anaphylaxis give rise to
characteristic changes in the pulmonary physiology. These will be discussed next.

Investigation of the effects on pulmonary physiology during anaphylaxis are often studied with lung mechanics. The parameters most commonly reported are pulmonary resistance (RL) and dynamic compliance (Cdyn). RL is a measure of the resistance of airways to airflow, and is mostly derived from large airways bronchoconstriction. Cdyn is a measure of the elasticity of the lung, and indicates peripheral airway involvement. Numerous investigators have found that during anaphylaxis RL increases and Cdyn decreases (110-115). The initial externally visible response to antigen challenge is an increased frequency of breathing with a decreased tidal volume (114). From this phase, laboured breathing with marked retraction of the thoracic wall follows (111). The bronchoconstriction produces other changes in lung function parameters. The functional residual capacity and residual volume both increase due to air trapping within the lungs (111). Another study of pulmonary mechanics in guinea pigs was designed to allow comparison with the measurements routinely used in studies of human asthma. Upon exposure to antigen, sensitized guinea pigs exhibited a decrease in the peak expiratory flow rate, the maximal mid-expiratory flow rate, the forced vital capacity, and the forced expiratory volume in 0.1 sec. This is similar to the acute response in asthmatic humans except that the forced expiratory volume over 1.0 sec. is assessed in humans, since humans have a longer duration of expiration. Furthermore, the forced expiratory volume in 0.1 sec. as measured by this group was not
preceded by a maximal inspiration to total lung capacity as is the usual method (116). An experiment in dogs has shown that during anaphylaxis there is a decrease in the arterial \( pO_2 \), pH, and heart rate (113). It can be seen, then, that the mediators of anaphylaxis have extensive detrimental influences on the efficiency of gas exchange in the lungs and that many studies have been undertaken to expand our understanding of the pathogenesis of asthma.

A variety of animal models have been described. Among these are dogs (10, 117), rabbits (45, 61), rats (58, 118), mice (119-121), monkeys (39, 122, 123) and calves (124). The most commonly used animal in studies of anaphylaxis, however, is the guinea pig (34, 92, 111, 115, 116, 125). There are a number of similarities between guinea pigs and humans which has made them the animal of choice. The first is that the lung is the major "shock" organ and source of mediators during anaphylaxis in both humans and guinea pigs (80). Furthermore, the mechanical properties of the lungs in guinea pig experimental asthma are similar to the mechanics in human bronchial asthma, both showing a decrease in \( C_{dyn} \) and an increase in \( R_L \) during anaphylaxis (81). In another study in guinea pigs undergoing anaphylaxis the pulmonary mechanics were evaluated in spontaneously breathing, anaesthetized guinea pigs forced by external pressure to perform an inspiratory/expiratory manoeuvre. This allows comparison of results with those from human studies. The respiratory rate increased, the peak expiratory flow rate and maximum mid-expiratory flow rate fell 20 %, forced vital capacity fell 50 %, forced expiratory volume in 0.1 sec fell 40 %, and
the use of aminophylline reversed these responses. These are similar to results from acute asthmatic humans (90). The predominant antibody mediator of anaphylaxis in humans is IgE, with some appearance of an IgG (51), while in guinea pigs there are IgG₁ and IgE-like antibodies involved (57). The SRS-A produced by antigen challenge of passively sensitized human lung and actively sensitized guinea pig lung were found to be pharmacologically, chemically, and chromatographically indistinguishable (104). The response of both tissue types to antigen-induced mediators is an immediate, short-lived contraction, followed by a sustained contraction. Antagonism of this airway smooth muscle contraction with diphenhydramine, an H₁ antagonist and FPL 55712, an SRS-A antagonist inhibited the initial fast contraction and subsequent slow contraction in both guinea pigs and humans (99). Other mediators are known to be released upon antigen stimulation, such as prostaglandins. Both human and guinea pig peripheral lung tissues respond to H₁ stimulation by producing PGF₂α and PGE. H₂ stimulation, and stimulation of parenchymal tissue with KCl or carbachol did not provoke PG generation. Stimulation of airway smooth muscle contraction with histamine, KCl, and carbachol caused the generation of PGE in humans, and PGE and PGF₂α in guinea pigs (106). So, while there are some differences apparent in the guinea pig and human systems, the basic similarities coupled with the ease of sensitization and ease of handling guinea pigs has made them a practical and popular animal model for the study of human allergic asthma.
Many approaches have been used by different investigators to produce an anaphylactically responsive guinea pig, with variations in the regimes of antigen immunization, challenge, and sensitivity assessment. Nares dusting with ragweed pollen or a distillate of ragweed pollen has been used to sensitize guinea pigs (126), as have intramuscular injections of 5% (w/v) ovalbumin (114, 127). The most common sensitization procedure is an intraperitoneal injection of antigen, most often ovalbumin, with or without an adjuvant (57, 110, 111, 128, 129). Passive sensitization with homologous antibody is also a successful method (115). Challenge via an antigen aerosol is common (110-112, 114-116, 126-129) although intravenous or subcutaneous challenges may also be used (57). The assessment of the level of anaphylactic response to antigen exposure has been measured as the length of time before onset of dyspnea (microshock time) (126-128) changes in Cdyn and RL (57, 110-112, 114, 115), PCA reactions (57, 126, 130) and in vitro responses of tissue to antigen and drugs (129).

Studies which have involved repeated challenges of guinea pigs over a period of time have shown that the high level of anaphylactic reactivity observed on initial antigen exposures diminishes with the continuation of antigen exposure (34, 111, 115, 126-128, 130). This phenomenon has been explored by a number of investigators, but no satisfactory explanation of this apparent induction of tolerance in the guinea pig has been found.

The study of this property of tolerance development is of interest because of a possible application to the human situation.
Although the characteristics of human asthma have been well described, the pathogenesis is uncertain. These observations of guinea pig tolerance induction lead to the hypothesis that human asthma arises due to a failure of the mechanism which operates in guinea pigs which become tolerant. The approach to this problem was to use guinea pigs sensitized with ovalbumin, which is an inexpensive, effective antigen and heat-killed Bordetella pertussis as an adjuvant since it has been shown in rats to preferentially stimulate the synthesis of reaginic antibodies (120, 131, 132), perhaps by enhancing the IgE-binding ability of lymphocytes (133). The guinea pigs received daily aerosol challenges to mimic the situation of chronic human exposure.

Experiments were then conducted to compare the responses of animals no longer responsive in vivo to aerosol challenge --the "tolerant" guinea pigs, with those of sensitized, responsive guinea pigs.

A summary of the experimental design (Fig. 2.1) is given to orient the reader to the overall plan and sequence of studies.
Figure 2.1. Summary of Experimental Design

- **IMMUNIZED**
  - 1 mg/ml OA aerosol
  - 2 weeks
  - Reactivity Assessment:
    a) Visual Grade
    b) Lung Mechanics
  - 1 hr/day
  - 5 days/week
  - 6 weeks
  - Aerosol Challenge Lay-Offs: 4, 6, 8, 10, 24 days

- **CONTROL**
  - 1 ml of 1 mg/ml OA & 1 ml of B. pertussis intraperitoneal

In Vitro Smooth Muscle Study
Total Histamine Assay
Passive Cutaneous Anaphylaxis
Lymphocytes transferred from Tolerant and Control animals to subsequently sensitized animals
Antigen-Induced Histamine Release from Chopped Lung
CHAPTER 2. METHODS AND MATERIALS

Part 2.1: Development and Assessment of Tolerance

2.1.1) Sensitization

Thirty 250 g male and female Hartley strain guinea pigs (outbred albinos) were injected intraperitoneally with 1 mg ovalbumin (OA) (45,000 M. Wt.) (Grade III, Sigma Chemical Co.) in 1 ml saline and 1 ml heat-killed Bordetella pertussis vaccine (HKP) (Connaught Laboratory).

2.1.2) Challenge Procedure

Two weeks after sensitization the animals were placed in a Plexiglass aerosolization chamber and exposed to a solution of $2.2 \times 10^{-5}$ M OA in saline nebulized at a flow rate of 5 l compressed air/min. from a Hudson disposable nebulizer. The aerosol challenge was performed for one hour each day, five days/week, for six weeks.

2.1.3) Assessment of Reactivity

2.1.3.1) Visual Grade

With every exposure to the aerosol the bronchoconstrictive response of each guinea pig was graded. This was based on a visual
assessment of the rate and depth of breathing and the degree of cyanosis. When no response was apparent a grade of zero was assigned. Rapid shallow breathing received a grade of one, slow laboured breaths with cyanosis received two, and a severe bronchoconstrictive response leading to collapse and rescue of the animal received a grade of three.

2.1.3.2) Lung Mechanics Assessment

During each of the six weeks of chronic aerosol exposure, six to eight animals from the group of 30 guinea pigs were randomly chosen for lung mechanics measurements. These animals were not challenged in the aerosolization chamber prior to these studies, but were anaesthetized with 20 mg/kg of sodium pentobarbital intraperitoneally and placed prone in a pressure sensitive guinea pig body plethysmograph. A pressure transducer (Validyne MP45 + 2 cm H2O) detected changes in box pressure and yielded a volume signal (T.V.). A saline-filled esophageal catheter with multiple side holes measured pleural pressure, and a differential pressure transducer (Hewlett Packard 267BC) which compares pleural and atmospheric pressure produced a transpulmonary pressure reading (PL). The tidal volume and transpulmonary pressure were recorded by a temperature-sensitive recorder (Hewlett-Packard 7758B), which also differentiated tidal volume to produce a flow signal. From the simultaneous readings of tidal volume, flow and transpulmonary pressure calculations of pulmonary resistance (RL) and dynamic compliance (Cdyn) were made (134). At points of zero flow the volume change (T.V.) was divided by PL to determine Cdyn. At points of
equal volume PL was divided by the flow to determine RL.

Aerosols of OA were administered via a funnel-like apparatus that directed the spray around the head of the guinea pig. Aerosols were given in 10-fold increases of concentration ranging from $10^{-12}$ to $10^{-7}$ M OA. Measurements were taken after a control saline aerosol, then one minute and four minutes after the start of each 30 sec. OA aerosol. Dose-response curves of RL and Cdyn versus nebulized concentration were constructed.

2.1.4) Investigation of the Stability of Tolerance

Following the six weeks of chronic OA exposure the animals were divided randomly into groups and withheld from OA challenge for a varying number of days (Lay-offs) to investigate the stability of tolerance when the animals are not continually exposed to the antigen. One group of four animals served as controls and were maintained on the five days/week schedule, while other groups of four, five, four, five and four animals were not aerosolized for 4, 6, 8, 10, and 24 days respectively. After the period of non-exposure the responses were assessed by both the visual grading system and by RL and Cdyn determined from lung mechanics.
Part 2.2: Investigations of the Mechanism of Tolerance

2.2.1) In Vitro Airway Smooth Muscle Studies

To test for in vitro antigen responsiveness, ten of the 30 animals which had been chronically exposed to antigen (T) were compared with ten sensitized weight-matched animals (S) and ten non-sensitized weight-matched controls (C). The animals were anaesthetized with 60 mg/kg sodium pentobarbital intraperitoneally and exsanguinated by cardiac puncture. The serum was separated and stored at -20°C for later comparison of antibody levels. The lungs and trachea were rapidly removed and placed in oxygenated Krebs' solution (NaCl, 95 mM; KCl 4.7 mM; MgSO$_4$, 2.3 mM; CaCl$_2$, 2.5 mM; KH$_2$PO$_4$, 1.2 mM; NaHCO$_3$, 25 mM; and anhydrous dextrose 12 mM). A strip of parenchyma (Par) was removed from the periphery of the right lung for the smooth muscle study, and the remainder of the right lung frozen at -20°C for a later histamine assay. All excess connective tissue and fat were removed from the trachea and bronchi. The trachea was cut in half to form upper tracheal (UT) and lower tracheal (LT) segments, and the bronchi (Br) dissected free of lung and separated from the trachea. These tracheal segments and the bronchi were spiralled by placing a thin wire rod through the lumen and cutting at an angle while gently pulling on one end of the tissue (135). The two tracheal spirals, bronchial spiral and parenchymal strip were placed in 20 ml organ baths...
containing Krebs' solution continuously bubbled with 95% O₂/5% CO₂ (v/v). The tracheal and bronchial strips were stretched by applying an initial weight of 2 g and the parenchyma stretched with an initial weight of 0.6 g. Following an equilibration period of approximately 60 min., cumulative dose-response curves to histamine dihydrochloride (Fisher Scientific) were performed. 0.1 ml aliquots of agonist were added to the baths to give final histamine base bath concentrations of \(5 \times 10^{-7}\) M, \(5.5 \times 10^{-6}\) M, and \(5.5 \times 10^{-5}\) M. Following thorough washing to return the tissues to their baseline state of contraction, the tissues were challenged with 10-fold increases in concentrations of OA ranging from \(1.1 \times 10^{-12}\) M to \(1.2 \times 10^{-7}\) M. Lastly, a large excess of carbachol (\(2.2 \times 10^{-4}\) M) (Carbamylcholine chloride, Sigma Chemical Co.), a cholinomimetic, was added to elicit the maximum tissue contraction. Contractions were measured using force-displacement transducers (Grass Model FT03C) calibrated to reflect developed force in g/9.8 m/sec². A four channel polygraph (Beckman Dynograph R511 A) recorded the raw data.

Contractile responses of the tissues were analyzed by plotting tissue responses in grams versus log Histamine and versus log OA and by plotting the percentage of the maximum histamine or carbachol response versus log Histamine and log OA.
2.2.2) Total Parenchymal Histamine Assay

The thirty lungs stored at \(-20^\circ C\) were thawed and two 0.5 g samples taken from each of the lungs. The samples were minced finely with scissors and placed in 10 ml test tubes with 2 ml PIPES buffer (25 mM PIPES, 120 mM NaCl, 5 mM KCl, pH 7.4). After boiling for 10 min. in a water bath the tissue was pelleted by centrifuging 15 min. at 1300 xg (Beckman TJ-6 centrifuge, TH-4 rotor) then 1 ml aliquots of the supernatant were removed and frozen at \(-20^\circ C\). The following day the histamine contents of the samples were assayed fluorometrically (Aminco Bowman SPF 124) as previously described (70) using the micro-modification of May et al (136) of the method described by Shore et al (137). Results were expressed as μg histamine/g tissue.

2.2.3) Passive Cutaneous Anaphylaxis (PCA)

2.2.3.1) Six hour sensitization

PCA's were performed according to the method of Ovary (138). Briefly, six female Hartley guinea pigs weighing approximately 300 g each were lightly anaesthetized prior to being shaved with an Oster small animal clipper (# 40 razor). Sera stored from the tolerant, sensitized and control animals used in the airway smooth muscle study were used. Sera from four animals in each of the three groups were pooled, then dilutions of 1/50, 1/100, 1/200, and 1/400 in saline were
made on the tolerant and sensitized sera. Two hours after shaving 0.1 ml injections of the dilutions of tolerant and sensitized sera, saline, and undiluted control serum were given, making a total of 10 dorsal intracutaneous injections per animal. After the six hour sensitization period 13 mg/kg intraperitoneal sodium pentobarbital and subcutaneous xylocaine (Astra Chemicals Ltd.) were administered. One ml of a solution of 10 mg/ml Evans blue dye (J. T. Baker Chemical Co.) and 2.2 x 10^{-6} M OA in saline was injected via a carotid cannulation. After 45 min. the guinea pigs were killed with an overdose of sodium pentobarbital and the skin removed, stretched and covered with borax. Twenty hours later the borax was brushed away and the long and short axes of the blue ellipses measured with vernier calipers to allow calculation of the area (πab) of each of the blue ellipses.

2.2.3.2) Four day and seven day sensitizations

Four and seven day sensitizations were done by the method of Watanabe and Ovary (55) and Parish (54). Seven days before the OA challenge four 300 g female Hartley guinea pigs were injected intracutaneously on one side of the back with unheated, sensitized and tolerant sera (same pooled sera as for six hour sensitization) at dilutions of 1/2, 1/4, and 1/8. Four days before the OA challenge heated (56°C for 30 min.) sensitized and tolerant sera were injected on the other side of the back in dilutions of 1/50, 1/100, and 1/200. The remainder of the experiment is as described for the six hour sensitization.
2.2.4) Lymphocyte Transfers

Four tolerant and four non-sensitized (Control) Strain 13 guinea pigs (inbred to allow cell transfers between animals) weighing approximately 1 kg were killed with 100 mg/kg sodium pentobarbital intraperitoneally. A modification of the technique described by Ford (139) was employed. The spleen, three to four hilar lymph nodes and three to four cervical lymph nodes were removed and placed in room temperature, pH 7.3, 0.73 % (w/v) NaCl/Phosphate buffered saline/acid citrate dextrose (PBS composition: $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3.21 mM; $\text{KH}_2\text{PO}_4$, 6.0 mM; ACD composition: anhydrous citric acid, 6.5 mM; sodium citrate, 7.7 mM; anhydrous dextrose, 10 mM). Excess fat and connective tissue were removed and the spleen and lymph nodes thoroughly rinsed before being finely minced with scissors. The tissues were gently pressed through a fine mesh sieve (W. S. Tyler Type 304, 100 μm grid) and the spleen and lymph node cells collected in NaCl/PBS/ACD. The 40 ml of spleen cell suspension were divided among 8 plastic conical centrifuge tubes, each with 30 ml distilled H$_2$O to lyse the RBC. After mixing for 30 sec. the tonicity was reconstituted with 15 ml of pH 7.3, 2.5 % (w/v) NaCl/PBS/ACD. The pellet from a 10 min., 22°C 200 xg (Beckman TJ-6, TH-4 rotor) centrifugation was resuspended in 20 ml 0.73 % NaCl/PBS/ACD. Each of the 20 ml lymph node and spleen cell suspensions were layered onto 8 ml of Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuged 30 min. at 22°C at 400 xg. The upper clear layer and pellet were discarded and the middle white
fluffy layer washed twice in 0.73 % (w/v) NaCl/PBS/ACD, then resuspended in 1 ml saline. Cells were counted in a haemocytometer using 2 % (v/v) acetic acid/methylene blue and viability assessed using Trypan blue exclusion. Cell viability was approximately 94 %. Total cells injected ranged from 0.57-2.2 x 10^8 cells.

Each of the eight Strain 13 recipients were injected intraperitoneally (140) with the combined spleen and lymph node cells from one donor. Approximately 24 hours later the guinea pigs were sensitized with OA as previously described, then aerosol challenged two weeks later.

2.2.5) In Vitro Histamine Release

Eight sensitized, chronically challenged Strain 13 guinea pigs (T), eight guinea pigs sensitized 14 days prior to the study but never challenged (S) and two non-sensitized, non-challenged control (C) guinea pigs were used in this experiment. The animals were heavily anaesthetized with approximately 100 mg/kg sodium pentobarbital intraperitoneally, then exsanguinated by cardiac puncture. The in vitro histamine release methodology of Austen and Brocklehurst was followed (141). The lungs and heart were removed en bloc, and the lungs flushed with 37°C Tyrodes solution (NaCl, 13.7 mM; CaCl₂·2H₂O, 0.14 mM; KCl, 0.27 mM; MgCl₂·6H₂O, 0.10 mM; NaH₂PO₄·H₂O, 0.04 mM; NaHCO₃, 1.2 mM; anhydrous dextrose, 0.56 mM) via the pulmonary artery until the blood was completely
cleared. Any portions of lung still left with blood were removed. The lung parenchyma was finely minced, then washed with 22°C Tyrodes solution via suction filtration. Eight tissue samples of 0.5 g were obtained from each lung and placed in 10 ml glass test tubes. Two ml of Tyrodes solution for control tubes and Tyrodes/OA in appropriate concentrations were added to the duplicate tubes. Four tolerant guinea pig and 4 sensitized guinea pig lung samples received OA in concentrations of $2.2 \times 10^{-9}$ M, $1.1 \times 10^{-8}$ M, and $1.1 \times 10^{-7}$ M. The remaining 4 tolerant, 4 sensitized, and 2 control animals received OA in concentrations of $1.1 \times 10^{-6}$ M, $1.1 \times 10^{-5}$ M and $1.1 \times 10^{-4}$ M. The tissues were incubated with antigen for 15 min. at 37°C with intermittent swirling. The supernatant from a 15 min., 4°C 1300 xg (Beckman TJ-6, TH-4 rotor, TJ-R refrigeration unit) was removed and stored at -20°C. The tissue pellet was resuspended in 2 ml Tyrodes solution and boiled 10 min. Following a centrifugation as previously described, 1 ml aliquots of the supernatant were removed and stored at -20°C. The histamine assay was carried out as described in the Total Parenchymal Histamine Assay section. Histamine released from tubes containing only Tyrodes solution was subtracted before calculation of histamine release in response to OA. The results were expressed as percentage histamine release versus OA concentration.
CHAPTER 3. DATA ANALYSIS

In the analysis of the lung mechanics data a one-way analysis of variance (ANOVA) (142, 143) was performed on Cdyn and RL using weeks as a grouping factor. This analysis was performed at each OA concentration. This method was chosen since it was desirable to find differences between an RL or Cdyn response between experimental weeks at a given OA concentration. The RL and Cdyn values for each animal were actually a percentage of a control measurement made on each animal. Expression of results in this way allows removal of some inter-animal variation. The control measurement was recorded each week prior to an OA dose. Upon completion of the ANOVA a significant difference between some groups was indicated. A Duncan's multiple range test (144) was then used to determine the weeks which were different.

The aerosol lay-offs assessed by a visual grade were compared using an unpaired T-test (144). The aerosol challenge lay-off data from lung mechanics was analyzed using Dunnett's T-test (144) since this test compares several groups to a control. In this experiment the day 43 Cdyn and RL served as controls while the days since lay-off were compared to them.

For the in vitro airway smooth muscle studies a one-way analysis of variance was performed on the mean histamine response as a percentage of the maximum response using control, sensitized and tolerant animals as the grouping factor. For this analysis UT, LT, and
Br were grouped together and analyzed. This was done since prior analysis by one-way ANOVA and Orthogonal Contrasts yielded no difference between these tissue types for a given group or concentration. Upon completion of the ANOVA, Duncan's multiple range test was again used to determine which groups were different. The above analysis was repeated for Parenchyma data.

The analysis of force increase per min. induced by histamine and OA was analyzed using a 3-factor analysis of variance. The factors were 1) dose level (i.e. histamine, OA) 2) group (C, S, T) and 3) subjects. This design, although complicated, allows the dose and group variations to be viewed after controlling for subject variation. It should be noted that the subjects are nested within the groups while grouping and dose level are fully crossed. This analysis was carried out for each of the four tissue types (UT, LT, Br, and Par). Orthogonal contrasts were used to assess differences between groups when significant results were obtained from the ANOVA.

The assessment of total histamine content was done using a two-way analysis of variance with subjects nested in groups (C, S, T). After a significant result Duncan's multiple range test was used.

For the PCA experiment the blue area was examined at each serum dilution by an unpaired T-test between S and T groups and all the blue areas in the S and T groups were compared using a three-way ANOVA. The large standard error of the mean is an indication of the large inter-animal variation.

The OA-induced histamine release was compared for S and T groups
at each concentration of OA using an unpaired one-tailed T-test. The test was done one-tailed since it could only be expected that the T value would be less than the S value. Although the standard error of the mean values varied from S to T groups the difference was not deemed sufficient to question the validity of the T-test. A three-way ANOVA was used to compare the S group to the T group.
CHAPTER 4. RESULTS

Part 4.1: Development of Tolerance

4.1.1) Assessment by Visual Grading System

Fig. 4.1 shows a plot of the daily mean visual grade of the group of 28 guinea pigs for the first 43 days of aerosol challenge. The first two weeks exhibit the highest responses with reactivity dropping off steadily thereafter. By 34 days tolerance to that regime of antigen challenge was established and no visible reaction to the aerosolized antigen could be detected. On the 35th day of challenge OA from a new bottle was administered. This provoked a markedly increased response for the next three challenges. This increased response was short-lived.

4.1.2) Assessment of Reactivity by Lung Mechanics

Figures 4.2 and 4.3 show the dose-response curves for dynamic compliance (Cdyn) and pulmonary resistance (RL) at 1, 22, and 43 days after the initial challenge. With increasing time of aerosol exposure higher doses of OA were required to elicit decreases in Cdyn and increases in RL. Comparison of Cdyn and RL on days 1, 22, and 43 at the OA concentration of $2.2 \times 10^{-4}$ M shows a significantly (ANOVA, Duncan's T-test p < 0.01) decreasing response (Table 4.1).
Figure 4.1. The daily mean visual grade ± SEM of 28-guinea pigs chronically challenged with OA aerosol for 43 days. See text for explanation of grading system.
Figure 4.2. OA-induced changes in lung mechanics during 43 days of chronic antigen aerosol challenge. Mean ± SEM % of Control Cdyn is shown for 1 day (N=6) (▲), 22 days (N=8) (◆) and 43 days (N=7) (●) of the chronic aerosol challenge period. Cdyn significantly different (p<0.01) at 2.2 x 10^{-4} M OA comparing each of days 1, 22 and 43 to the others.
Figure 4.3. OA-induced changes in lung mechanics during 43 days of chronic antigen aerosol challenge. Mean $\pm$ SEM % of Control RL is shown for 1 day (N=6) (Δ), 22 days (N=8) (◆) and 43 days (N=7) (●) of the chronic aerosol challenge period. ◆ RL significantly different ($p<0.01$) at $2.2 \times 10^{-4}$ M OA comparing each of days 1, 22 and 43 to the others.
Table 4.1. Lung Mechanics Assessment of Tolerance Development

<table>
<thead>
<tr>
<th>Time During Chronic Exposure</th>
<th>Mean % Decrease Cdyn $\pm$ SEM at 2.2 x $10^{-4}$ M OA</th>
<th>Mean % Increase RL $\pm$ SEM at 2.2 x $10^{-4}$ M OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>79 $\pm$ 10 $^*$</td>
<td>450 $\pm$ 200 $^*$</td>
</tr>
<tr>
<td>N=8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 22</td>
<td>40 $\pm$ 8 $^*$</td>
<td>87 $\pm$ 50 $^*$</td>
</tr>
<tr>
<td>N=6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 43</td>
<td>11 $\pm$ 6 $^*$</td>
<td>40 $\pm$ 14 $^*$</td>
</tr>
<tr>
<td>N=8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^*$ Significantly different from each of the other days ($p < 0.01$)
4.1.3) Effect of Challenge Lay-Offs on Tolerance

The effects of challenge lay-offs on tolerance were assessed visually and by lung mechanics. Fig. 4.4 compares the effects of 4 through 24 days lay-off with the mean visual grade for those guinea pigs regularly challenged. Increased reactivity was observed for the 10 day lay-off period (unpaired T-test p<0.05).

Table 4.2 compares the mean % of control ± SEM Cdyn and RL at $2.2 \times 10^{-4}$ M OA for 4, 6, 8, 10 and 24 days lay-off. Some groups (4 and 8 days) recover significant reactivity (Dunnett's T-test p<0.05), but never regain their initial levels. Increasing lay-off time does not result in a progressive loss of tolerance.

Part 4.2: Investigations of the Mechanism of Tolerance

4.2.1) In Vitro Airway Smooth Muscle Studies

Fig. 4.5 illustrates sample UT tolerant and sensitized recorder tracings of a complete experiment. Tissue contractions began within one min. of the addition of agonist to the organ bath.

Regardless of the group or drug used to provoke contraction, the magnitude of grams response induced was largest for UT, slightly less for LT, slightly less again for Br, and much less for Par (Fig. 4.6, 4.7). However, when responses were expressed as a percentage of the maximum tissue contraction induced by carbachol or histamine, the UT,
Figure 4.4. The weekly mean visual grade ± SEM of 26 guinea pigs challenged and withheld from challenge over a period of 20 weeks. Four animals served as controls and were maintained on the 5 days/week challenge schedule, while other groups of 4, 5, 4, 5 and 4 animals were laid off challenge for 4 (△), 6 (○), 8 (■), 10 (▲), and 24 (●) days respectively. (X) represents the mean response of the group of guinea pigs aerosol challenged for one week. ❇ Significantly different from Control after 10 days lay-off (p<0.05).
<table>
<thead>
<tr>
<th>Time</th>
<th>Mean % of Control Cdyn ± SEM at 2.2 x 10^{-4} M OA</th>
<th>Mean % of Control RL ± SEM at 2.2 x 10^{-4} M OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 Chronic Exposure</td>
<td>22 ± 10</td>
<td>550 ± 200</td>
</tr>
<tr>
<td>Day 43 Chronic Exposure</td>
<td>89 ± 6</td>
<td>140 ± 14</td>
</tr>
<tr>
<td>4 days lay-off</td>
<td>49 ± 13 *</td>
<td>190 ± 34 *</td>
</tr>
<tr>
<td>6 days lay-off</td>
<td>80 ± 16</td>
<td>110 ± 30</td>
</tr>
<tr>
<td>8 days lay-off</td>
<td>43 ± 17 *</td>
<td>210 ± 49 *</td>
</tr>
<tr>
<td>10 days lay-off</td>
<td>65 ± 14</td>
<td>160 ± 30</td>
</tr>
<tr>
<td>24 days lay-off</td>
<td>61 ± 15</td>
<td>160 ± 23</td>
</tr>
</tbody>
</table>

* Significantly different from Day 43 (p < 0.05).
Histamine, OA, and carboclool.

Sensitive and tolerant Ut tissue when exposed to the agonists

Figure 4.5. Sample dose-response tracking from representative

Histamine (M)  Carboclool (M)

10-12 10-11 10-10 10-9 10-8 10-7 2 x 10-7

Max

Wash

Tolerant

Max

Wash

Sensitive
Figure 4.6. Mean grams response ± SEM to histamine and carbachol by control tissues UT (N=10) (●), LT (N=10) (◇), Br (N=10) (◆), and Par (N=10) (□).
Figure 4.7. Mean grams response ± SEM to ovalbumin by sensitized tissues UT (N=10) (●), LT (N=10) (◆), Br (N=10) (×), and Par (N=10) (■).
Figure 4.8. Mean % of maximum histamine response to histamine by control tissues UT (N=10) (●), LT (N=10) (◆), Br (N=10) (×) and Par (N=10) (■).
Figure 4.9. Mean % of maximum carbachol response ± SEM to ovalbumin by sensitized tissues UT (N=10) (●), LT (N=10) (◆), Br (N=10) (◆) and Par (N=10) (■).
LT and Br responses were similar, but Par remained distinct (Fig. 4.8, 4.9). For this reason, UT, LT and Br data are combined for comparing control, tolerant and sensitized groups.

Fig. 4.10 compares the responses of control, sensitized and tolerant tissues to increasing doses of histamine. No difference in airway smooth muscle contractility between the groups can be detected.

Comparison of rates of OA-induced tissue contraction show that except for tolerant bronchi, which had a significantly greater (ANOVA p<0.01) maximum rate of contraction (0.36 ± 0.04 g/min.) than sensitized bronchi (0.13 ± 0.04 g/min.) rates of force generation were similar for sensitized and tolerant tissues. Maximum rates of contraction of sensitized tissues were elicited at mid dose-response curve concentrations and for tolerant tissues at upper dose-response curve concentrations.

All contractions were sustained and required several hours of washing before the tissues relaxed to the baseline state. Repetition of the OA dose-response curve produced contractions which were greatly reduced or non-existent.

Fig. 4.11 compares control, sensitized and tolerant airway smooth muscle responses to OA. Lack of control smooth muscle response to OA shows that OA does not cause any non-specific contraction. Comparison of tissue from sensitized animals with tissue from tolerant animals shows a significantly decreased response by the tolerant tissue over the OA dose range studied (ANOVA, Duncan's T-test p<0.001 at 1.2 x 10^-10 M OA to 1.2 x 10^-7 M).
Figure 4.10. Mean % of maximum tissue response ± SEM to histamine by Control, Tolerant and Sensitized groups. UT, LT and Br (— — —) data have been combined so that N=30 for each of Control (■), Sensitized (▲), and Tolerant (●) groups. N=10 for Par tissue (— — —) in each of Control (■), Sensitized (▲), and Tolerant (●) groups.
Figure 4.11. Mean % of maximum tissue response + SEM to ovalbumin by Control (■), Tolerant (●), and Sensitized (▲) groups. UT, LT and Br data have been combined so that N=30 for each of the groups.

☆ Significant decrease (p < 0.001) in response by tolerant tissue over the OA dose range $1.2 \times 10^{-10}$ M to $1.2 \times 10^{-7}$ M.
4.2.2) Total Tissue Histamine Content

Table 4.3 shows the results for the lung tissue histamine content in tolerant, acutely sensitized and control guinea pigs. The tolerant group of animals had significantly (ANOVA, Duncan's T-test \( p < 0.05 \)) more histamine (5.74 \( \pm \) 0.76 \( \mu \)g histamine/g wet tissue) than the control (3.05 \( \pm \) 0.33) or sensitized (3.30 \( \pm \) 0.38) groups.

4.2.3) Passive Cutaneous Anaphylaxis

Fig. 4.12 shows the PCA reaction from a guinea pig which had a six hour sensitization with serum antibody. No response was elicited by saline or undiluted control serum. Comparison of the areas of blueing from tolerant and sensitized sera shows no grossly apparent significant difference in antibody levels in sera from tolerant and sensitized animals.

Table 4.4 presents the mean areas of reaction (mm\(^2\)) of each serum dilution from tolerant and sensitized sera. Comparison of the sensitized with the tolerant group over all the concentrations shows the tolerant group to have significantly less area of blueing than the sensitized group (ANOVA \( p < 0.05 \)). By an unpaired T-test, the groups were the same except at the 1/100 dilution (\( p < 0.05 \)).

The results from the four 48 hour and seven day sensitizations were impossible to quantify, since systemic sensitization of the animals occurred, and only two animals survived the antigen challenge.
Table 4.3. Total Lung Parenchymal Histamine

<table>
<thead>
<tr>
<th>Tissue</th>
<th>μg histamine/g wet lung ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.05 ± 0.33</td>
</tr>
<tr>
<td>Sensitized</td>
<td>3.30 ± 0.38</td>
</tr>
<tr>
<td>Tolerant</td>
<td>5.74 ± 0.76 *</td>
</tr>
</tbody>
</table>

* Significantly different from Control and Sensitized (p < 0.05).
Figure 4.12. Comparison of sera from sensitized and tolerant guinea pigs by a six hour passive cutaneous anaphylaxis sensitization.
Table 4.4. Six Hour PCA Comparison of Sensitized and Tolerant Sera.

<table>
<thead>
<tr>
<th>Serum Dilution</th>
<th>Area of Blueing mm² ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitized</td>
</tr>
<tr>
<td>1/50</td>
<td>364 ± 35</td>
</tr>
<tr>
<td>1/100</td>
<td>222 ± 20</td>
</tr>
<tr>
<td>1/200</td>
<td>164 ± 20</td>
</tr>
<tr>
<td>1/400</td>
<td>119 ± 27</td>
</tr>
</tbody>
</table>

* Groups significantly different from each other (P < 0.05)
to provide areas of blueing. The areas of blueing were more diffuse than the areas from the 6 hour sensitization, but approximate comparison of these areas did not show a reduced blueing reaction from tolerant sera. Unfortunately, more serum was not available, so the IgG\textsubscript{1b} and the IgE studies could not be repeated with PCA or an alternate assay method.

4.2.4) Lymphocyte Transfers

Table 4.5 shows the visual grade assigned to each of the lymphocyte recipients. All four guinea pigs which received lymphocytes from tolerant animals reacted with strong bronchoconstriction to the OA aerosol challenge. Three of the four animals which received lymphocytes from non-sensitized control animals reacted strongly.

4.2.5) In Vitro Antigen-Induced Histamine Release

Table 4.6 shows the results for the in vitro histamine release from tissue from acutely sensitized non-exposed animals and chronically exposed tolerant animals. The groups have a similar maximum release of histamine, with approximately 20 % antigen-induced release of histamine observed with the three highest OA concentrations. The release of histamine in response to \(1.1 \times 10^{-9} \text{ M OA}\) is significantly less (0.34 ± 0.27 \(\mu\)g histamine released/g tissue, 1.0 ± 0.7 % histamine released) for the tolerant group than for the sensitized group (1.2 ± 0.3 \(\mu\)g)
histamine released/g tissue, 5.1 ± 1.4 % histamine released) (unpaired T-test p < 0.05). Comparison of the two groups over all the OA concentrations using a three-way ANOVA shows no significant difference between the groups. These results are shown graphically in Fig. 4.13.
Table 4.5. Visual Grade Assessment of Lymphocyte Transfer Effect on Response to OA Challenge

<table>
<thead>
<tr>
<th>Lymphocytes Donated By:</th>
<th>Tolerant guinea pigs</th>
<th>Control guinea pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual Grade</td>
<td>2 2 2 2</td>
<td>2 2 2 0</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6. Ovalbumin-Induced Histamine Release from Chopped Guinea Pig Lung

<table>
<thead>
<tr>
<th>Group</th>
<th>Ovalbumin (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.1 x 10^{-9}</td>
</tr>
<tr>
<td>Sensitized</td>
<td>1.2 ± 0.3 *</td>
</tr>
<tr>
<td></td>
<td>(5.1 ± 1.4)</td>
</tr>
<tr>
<td>N=4</td>
<td></td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.34 ± 0.27a</td>
</tr>
<tr>
<td></td>
<td>(1.0 ± 0.7)b</td>
</tr>
<tr>
<td>N=4</td>
<td></td>
</tr>
</tbody>
</table>

a Values are mean µg histamine released/g wet lung ± SEM
b Values are mean % release of histamine ± SEM
* Significantly different from Tolerant tissue at this OA concentration (p<0.05)
Figure 4.13. Comparison of % histamine release induced by ovalbumin for Sensitized (N=4) (▲) and Tolerant (N=4) (●) groups.

● Significant difference (p<0.05) between the groups at 1.1 x 10^{-9} M OA.
CHAPTER 5. DISCUSSION

The development of a state of non-reactivity (tolerance) to antigen challenge in sensitized, chronically aerosol challenged guinea pigs has been noted previously (39, 125-127, 130, 145) and has in this series of experiments been further explored. For many, the goal of the use of guinea pig anaphylaxis is the development of a model of chronic human asthma. While the loss of an anaphylactic response to antigen challenge precludes the use of guinea pig anaphylaxis for this purpose, the understanding of the mechanism of this tolerance holds possibilities for interference and subsequent development of a chronic model. In addition, it may suggest other areas for investigating the etiology and treatment of human asthma.

Over the weeks of daily aerosol challenge several features of the guinea pig model became apparent. One of the most striking is the large variation in inherent reactivity among the animals. This large variation is present in both outbred Hartley guinea pigs and inbred Strain 13 guinea pigs. The animals could be classified as ranging from "high responder" to "low responder" guinea pigs (Fig. 5.1). Once they had developed a tolerant state, some animals occasionally had sporadic episodes of 'breakthrough' reactivity. These would only last one day, were not linked to the 'breakthroughs' by other guinea pigs and had no apparent temporal cycle.

There was also variation in the pattern of response in the first two weeks of antigen exposure. Roughly 50% of the guinea pigs...
Mean Visual Grade/Exposure

Figure 5.1. Thirty-two guinea pigs were assigned a visual grade for reactivity to chronic ovalbumin challenge over a period of five to seven weeks. The total of all grades was divided by the number of exposures to provide a distribution of reactivity among the group of animals. See text for explanation of grading system.
exhibited their strongest response in the first week of challenge, while the other 50% responded most strongly during the second week. This variation suggests that not all animals are rapidly thoroughly sensitized by the intraperitoneal OA/HKP injection. Those animals which do not react until the second week may be further sensitized by inhalation or just require a longer sensitization time to produce a visible response to challenge. Other investigators have successfully used aerosol delivery of antigen as a sensitization method (111, 126). Although intraperitoneal injection of antigen is most common (111, 127, 128), feeding of antigen (130) and passive sensitization (146) have also been used. Abdominal processing of antigen seems to occur in each of the active sensitization methods (45).

Aerosol challenge to mimic the conditions of provocation of human allergic asthma is the most common method of assessing the level of bronchoconstrictive reactivity. Depending upon the schedule of antigen exposure, the diminished reactivity in response to chronic aerosol challenge occurs as early as the second week of antigen exposure (111, 146) or as late as the fourth week (147).

During the course of repeated aerosol exposures it was observed that the length of time before onset of cyanosis and bronchoconstriction increased. In the first few challenges, responses were elicited within the first several minutes. After two weeks of challenge, numerous animals were taking as long as 40-50 min. before exhibiting their reaction. This phenomenon indicates the gradual onset of tolerance, and seems to be related to the dose or concentration of
the antigen being processed by the animal, since Paré et al (111) found that by increasing the concentration of OA in the aerosol the initial level of bronchoconstriction could be maintained. The longer the animals inhale antigen, the larger the concentration will be within the lungs. This apparent dose-dependency may involve a concentration gradient of antigen in the mucus, and/or competition for antigen-specific IgG\textsubscript{1} or IgE at the level of the mast cell. As tolerance develops, the minimum amount of antigen required to elicit a response increases, thus demanding more time and an increased OA concentration at the target site. There may also be an increased efficiency of removal of the antigen from the airway, perhaps by improved transport of the antigen-laden mucus.

As an objective means of assessing bronchoconstrictive reactivity lung mechanics were studied on the chronically challenged guinea pigs. This provided a corroboration for the development of tolerance as assessed by the visual grading.

The stability or lability of tolerance when antigen exposures are terminated is of interest. Hicks and Skeldon (128) have reported that when guinea pigs are desensitized by repeated challenge after a 30-60 day initial sensitization time, resensitization is impaired after a 7 day lay-off. Herxheimer (127) has reported that after desensitization, resensitization occurs to a variable degree depending upon the number of days without challenge and the individual guinea pig. In the aerosol lay-off experiments described here, withholding the aerosol for several days does not result in a uniform return to the sensitive
state. Responses from some of the 22 originally unresponsive animals which were withheld from OA exposure for 4-24 days do show an increased bronchoconstriction in response to the aerosol when assessed by either a visual grade or lung mechanics.

Some possible mechanisms for tolerance include decreased smooth muscle sensitivity to histamine, depletion of mediators in mast cells, altered mast cell function, or an immunologically mediated mechanism.

The in vitro airway smooth muscle study was undertaken to investigate the possibility of a decreased smooth muscle response to histamine in tolerant animals. Comparison of the forces elicited by contraction of the tissue segments at the maximal histamine dose showed these patterns (Fig. 4.6) in all of the groups (C, T, and S): UT ≥ LT; UT > Br, Par; LT = Br; LT > Par; Br > Par. These differences may be attributed to various tissue characteristics such as: decreasing smooth muscle in lower airways, more connective tissue in lower airways, changes in airway histamine receptor population, changes in $H_1$ receptor responsiveness, variations in size of tissue and variations in cutting of tissue.

Conversion of the data to percentage of maximal tissue response (usually elicited by carbachol but sometimes by maximum histamine dose) normalizes for these differences so that UT, LT and Br responses in all the groups become the same. The parenchyma, however, remains significantly different (ANOVA, Orthogonal contrasts p<0.05) from UT, LT and Br in all groups. At low histamine doses the parenchyma achieves a larger proportion of its maximum than do UT, LT and Br.
Possible reasons for this characteristic of apparently greater histamine sensitivity by Par may be an increased number of histamine receptors and/or increased $H_1$ receptor responsiveness, or larger numbers of $\alpha_1$ receptors (16). Another possibility is that there is less resistance to contraction from the lack of cartilage in parenchyma, so small smooth muscle contractions are more easily observed as a decreased tissue length. The critical conclusion from this study of in vitro smooth muscle response to histamine, however, is that the Tolerant, Sensitized, and Control groups display equal reactivity to histamine.

The second part of this experiment investigated the responses of the tissues to OA. The contractile responses obtained from each tissue of each group showed the features of the typical Schultz-Dale reaction (148). These are a rapid onset of response, sensitivity to low OA concentrations, sustained contractions requiring long washing to remove, and desensitization of the tissue to a second exposure to OA.

The rightward shift in the "tolerant" OA dose-response curve suggests a decreased mediator release from mast cells in response to the OA stimulus. The character of response to OA of the airway smooth muscle appears unchanged between tolerant and sensitized animals. The rates of contraction are the same for tolerant and sensitized groups except for the tolerant bronchial rate of contraction which is significantly greater (ANOVA, Orthogonal contrasts $p < 0.01$) than the sensitized bronchial rate of contraction. The relevance of this observation is uncertain since only the bronchial tissue segments
displayed this property. It may indicate that an anaphylactic reaction in guinea pigs is strongest in the bronchi due to altered $H_1$ receptors.

A common finding in human asthma is hyperplasia and hypertrophy of airway smooth muscle. These organ bath studies give indirect evidence that such airway smooth muscle changes have not occurred in the chronically OA-exposed, tolerant guinea pigs. This conclusion can be drawn from the fact that the maximum force generated by tolerant, sensitized and control animals is the same. Secondarily, the rates of contraction are similar except for the previously mentioned bronchial segments.

The possibility that tolerance may be due to decreased mediators in the lung was approached by comparing lung histamine contents of control, sensitized and tolerant animals. The emphasis put on histamine in these experiments does not intend to ignore the contribution of other mediators of anaphylaxis, but the actions of histamine are relatively well understood, it can be readily assayed, and it has been shown to be an important inducer of airway constriction in guinea pigs (149-151). The finding that histamine was not depleted in chronically challenged animals was also reported by Broder (146). However, in our group of tolerant Hartley guinea pigs, the histamine content was significantly greater than that of control and sensitized animals. This suggests that there may be a mechanism inhibiting the release of histamine.

Another possible non-immunologically mediated mechanism has been
investigated by McCaig and Souhrada (35). The resting membrane potential of guinea pig airway smooth muscle was observed to decrease with chronic inhalation of aerosolized albumin. In addition, airway smooth muscle depolarized to a lesser extent in response to histamine and OA as the period of chronic challenge increased. Present data is not compatible with such changes since the airway smooth muscle responsiveness to histamine was unchanged.

With no apparent physiologic mechanism having been shown, immunologic mechanisms were considered. Some possible explanations for the development of tolerance include a decrease in homocytotropic antibody levels, the presence of a blocking antibody, decreased antibody receptors on mast cells, or suppressor cells or factors acting to prevent mast cell mediator release.

Many other investigators have attempted to correlate the PCA titre with the level of anaphylactic response (52, 53, 126, 128, 130). These studies have shown that there is no correlation between the rapidity of the response and the PCA titre (126). Further, the absence or diminishment of a response does not correlate with a decreased PCA titre (126, 128, 130). The only positive correlation between PCA reactions and anaphylaxis is that when anaphylactic responses are present, so are PCA reactions. There are three homocytotropic antibodies known in the guinea pig. These are IgG_{1a} (152), IgG_{1b} (153) and reaginic (56, 154-156) antibodies. The presence of these antibodies can be detected by allowing different times for sensitization, and heating some serum samples. IgG_{1} antibodies are
stable to heating at 56°C for 1 hour and are present in mg/ml levels in the serum of guinea pigs (157). The IgG\textsubscript{1a} can be detected after a 4 hour sensitization period, but persists in the skin for only 1-4 days (54, 157) while IgG\textsubscript{1b} can be detected after 18 hours of sensitization (53) and persists for up to 7 days (53, 55) although becoming increasingly weak. IgE exists in only ng/ml quantities in serum, is labile to heating at 56°C for 1 hour, and persists in skin for several weeks (157). More than 18 hours is required for sensitization (53). The six hour sensitization therefore shows only IgG\textsubscript{1a} antibody, and the 4 day sensitization with heated serum shows only IgG\textsubscript{1b} antibody. The 7 day sensitization shows primarily the presence of IgE, although some contribution from residual IgG\textsubscript{1b} may be present. IgG\textsubscript{1a} was shown to be significantly decreased in tolerant guinea pig serum, thus providing a possible mechanism for decreased reactivity. Although there does not seem to be a large difference between the two groups, there is a consistent trend to slightly smaller areas of blue in the tolerant group. What this means in terms of actual differences in Ab titre is not known, and even if it were, the exact relationship between Ab quantity and mast cell release of mediators is unknown. If, for instance, all the Fc receptors are saturated at the level of Ab present in the tolerant group or at an even lower Ab level, mediator release would not be inhibited in the tolerant group, even though less Ab was circulating.

No direct assays for blocking antibodies were carried out, since experiments by Blanchard et al (126) indicated that although
precipitating antibodies were present in the serum of non-responsive animals, these did not block the PCA reaction.

Another possibility for an antibody-mediated mechanism of tolerance has been suggested by André et al (145) in their system of mice intragastrically immunized with SRBC. Their results indicate the formation of IgA immune complexes with enteric immunization. This produces a tolerogenic effect which can be transferred to virgin recipients by way of serum. Attempts at transferring serum from non-responsive guinea pigs to acutely sensitized guinea pigs has so far failed to inhibit the anaphylactic response (126). The possibility that IgA is formed and acts locally in the lung mucosa as a "blocking antibody" cannot be ruled out, however.

The presence of suppressor cells has been well documented in various systems (158-162) and its role in the development of immunological non-responsiveness has support in numerous systems. A sub-population of T-cells having a number of functions has been activated with ConA in vitro. The actions of this population of cells include suppression of antibody production (163, 164), suppression of cytotoxic lymphocyte generation (165, 166), suppression of T-cell mitogenic response (167) and suppression of mixed lymphocyte culture (MLC) (168). Miller et al (147) found that antigen-specific suppressor T-cells from OA-fed mice could induce suppression of humoral and CMI responses when transferred to normal recipients. Ngan and Kind (169) detected suppressor T-cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. A similar
finding has been reported by Chiorazzi, Fox and Katz (170) where IgE "non-responder" mouse strains were converted to IgE "responders" by the elimination of suppressor T-cell activity. The mechanism of action of suppressor cells is not completely understood, but the production of suppressor factors is a strong possibility. Katz and co-workers (171, 172) have detected a non-immunoglobulin "suppressive factor of allergy" (SFA) present in the serum and ascites fluids of CFA-inoculated low IgE responder mice. X-irradiation of low-responder mice near the time of primary immunization causes an increase in IgE production, and results in enhanced primary or adoptive secondary IgE antibody responses. Giving serum with SFA to irradiated mice abolished the irradiation-enhanced responses. Transfer of SFA sera to high responder mice, however, showed these mice to be relatively unaffected by the suppressive activity. Kontiainen et al (173) have also reported a suppressor factor produced in vitro which when injected about the time of KLH-antigen administration inhibits the in vitro IgM and IgG response to trinitrophenyl KLH. Of most interest, however, is the presence of a human suppressive factor of allergy which has been demonstrated by Katz (174) in an in vitro system in which addition of human SFA inhibited IgE antibody synthesis by human peripheral mononuclear cells.

A regulatory link between the mediators of anaphylaxis and the cells of the immune system has been detected by the discovery of lymphocytes which bind histamine (175-179). Suppressor cells generated by histamine have been shown to suppress the in vitro proliferative
response to antigen (MLC) and mitogen (180). Reports of lymphocytes bearing histamine receptors have shown that they also modulate in vitro release of histamine from basophils (181), lymphocyte-mediated cytotoxicity (182), antigen-induced MIF (183) and lymphocyte proliferation (184). Another mediator which appears to be involved in the stimulation of non-specific T suppressor cells is prostaglandin E (185-187). PGE is known to be present during anaphylaxis as a bronchial smooth muscle relaxer, so it appears that it may function in two ways to inhibit the anaphylactic response.

The presence of suppressor cells was not demonstrated in the protocol followed in this experiment. However, this is not proof that suppressor cells are not present. It is possible that a different protocol, perhaps using a different sensitization time, method of lymphocyte isolation, route of lymphocyte administration, method of activity assessment or irradiated recipients would allow detection of suppressor activity. The most obvious indicator of suppressor activity, however, would be decreased antibody production, and the results from PCA studies suggest that this is so. Perhaps data from IgG\textsubscript{1b} and IgE assays would support this. There may be as well, however, an alternative mechanism of suppressive activity. As previously described (181), lymphocytes bearing histamine receptors have been shown to inhibit histamine release from basophils in vitro. It is possible, therefore, that other cell types may be directly or indirectly influenced by a suppressive mechanism.

The study of in vitro antigen-induced histamine release from
chopped guinea pig lung suggests that there is a mechanism whereby the normal release of histamine from mast cells is inhibited in animals unresponsive in vivo to a given level of OA antigen aerosol. Mast cells from tolerant guinea pigs release significantly less histamine than acutely sensitized guinea pigs when exposed to a low concentration of OA. Less histamine is released both quantitatively and as a percentage release. At the time these experiments were carried out, the histamine released at the lowest OA concentration used was near the lower limit of the sensitivity of the histamine assay. For this reason, lower concentrations of OA could not be used. However, a new histamine assay with greater sensitivity is now being developed which would allow further studies at lower OA concentrations. Although only four guinea pigs were studied, the animal at the 'high responders' end of the distribution (> 0.71, Fig. 5.1) released appreciably more histamine (128 ng) at the lowest OA dose than those in the 'lower responders' portion of the distribution (0-0.40), (i.e. 2, 9, 14 ng histamine). This suggests that the antigen-induced histamine release from chopped lungs is a fairly sensitive assay of the degree of tolerance.

The calculation of total histamine content/g wet lung from the tissue used for antigen-induced histamine release produced values 6-10 fold larger than the previous total histamine assay. This placed the total histamine content from the Strain 13 group into the range reported by others (53). The reason for this difference is not clear, since the only difference between the two groups is that the first
experiment involved Hartley guinea pigs which had been aerosol challenged for 8-12 months and the second experiment involved Strain 13 guinea pigs which had been aerosol challenged for 9 months.

Rather than an inability to respond, tolerance appears to be an active inhibition of a system which is fully capable of action. Evidence from in vitro and in vivo studies support this. In the higher concentration range of OA-induced histamine release from chopped lung, the tolerant tissue is able to release as much histamine as the sensitive tissue. In vivo, Paré et al (111) showed that animals recovered their responsiveness if the concentration of inhaled antigen was increased progressively. Thus, the mechanism of tolerance is connected to the quantity of antigen normally present in the system. It seems, however, to require more than a simple increase of antigen in the system to overcome the mechanism of tolerance. This is suggested by an observation made during the period of chronic aerosol challenge. A group of tolerant guinea pigs was exposed to OA from a new bottle. The following day the majority of guinea pigs exhibited severe anaphylactic responses (Fig. 4.1). It is possible that the new bottle of OA (of a different Lot #) was delivering more antigen per weight than the old bottle, due to the handling of the old bottle, which predisposed to much absorption of water by the lyophilized OA. This suggests that some "processing" of the antigen must occur to equip the system to respond to the new level of antigen(s). There may also have been new or contaminating antigens in higher concentration in the new bottle which acted as a booster and stimulated a rapid response on the
following day. The fact that there wasn't a strong response on the day of initial exposure correlates with the hypothesis of an active mechanism of inhibition, rather than insufficient quantities of antigen for a given quantity of available antibody.

The experiments undertaken to explore the development of tolerance have confirmed observations made by others (39, 111, 125-127, 130, 145) that during chronic aerosol challenge, the bronchoconstrictive response of sensitized guinea pigs becomes progressively less rapid and less severe until eventually the animals are unreactive to the usual challenge procedure, i.e. a state of 'tolerance' arises. However, while many observations of this have been made in terms of lengthening 'microshock' or 'preconvulsion' time (52, 127, 128, 146) or in terms of pulmonary mechanics before and after antigen challenge (111, 115, 116), experiments have not previously been carried out which followed the gradual decrease in visible reactivity with the lung mechanics parameters of Cdyn and RL. This work has shown that Cdyn and RL are a good objective assessment of guinea pig changing anaphylactic responses to aerosol challenge.

The conclusion drawn from these experiments that withholding aerosol challenge produces only a variable resensitization confirms observations made by Hicks and Skeldon (128) and Herxheimer (127).

Findings from this work that airway smooth muscle from tolerant animals is as responsive to histamine-induced contraction but less responsive to ovalbumin-induced contraction than sensitized animals was first reported in an abstract (188). This definitively eliminates the
possibility that 'tolerance' is due to decreased airway smooth muscle response to histamine. This has since been confirmed by Hedman and Andersson (189).

The studies of histamine content in the lung parenchyma of tolerant, sensitized and control guinea pigs has shown a tendency for tolerant animals to have higher levels of histamine, thus decreasing the likelihood of mediator depletion being the mechanism of tolerance. No other reports of this phenomenon have been made.

Some investigators have reported a poor correlation between level of reactivity and PCA (126) or haemagglutinating (128) results. Others have reported that unreactivity is associated with a decrease in IgG as assessed by PCA (130, 190). This data is supportive of the latter conclusion.

Antigen-induced histamine release was observed in these experiments to be decreased in Tolerant chopped lung tissue when a low level of antigen was used. An experiment by Hedman and Andersson (189) comparing antigen-induced histamine release between tolerant and sensitized guinea pigs showed no differences in release at an OA concentration of $2 \times 10^{-7}$ M. This is not unexpected, as data shown here indicates that histamine releases are comparable at high levels of OA.

These experiments suggest that quantitatively less mediators are released in the tissue from tolerant animals or that there is a decreased tissue response to released mediators other than histamine. In support of the latter hypothesis, Hedman and Andersson (189) have
reported that in vitro responses of trachea from animals desensitized by a 7 day regime of OA injections exhibited a contractile response to SRS-A which was 38% of that shown by tracheas from sensitized animals.

However, this does still not exclude the possibility that upon antigen challenge less histamine and SRS-A could be released from the tissue.

A mechanism acting at the level of the mast cell provides a possible explanation for the data. One such mechanism would be a decrease in the number of Fc receptors. This is a non-specific mechanism, and would interfere with the reactivity of mast cells in response to an antigen to which the guinea pig had just been sensitized.

Another mechanism would be a direct suppression of mast cell mediator release by a suppressor cell-mast cell contact, or via a specific or non-specific soluble suppressor factor acting on the mast cell membrane. Given that a histamine-induced suppressor cell is known to inhibit histamine release from basophils (181) this is a distinctly possible mechanism.

In conclusion, the tolerance observed in guinea pigs chronically exposed to an aerosol of OA antigen appears to manifest at least in part by an inhibition of antigen-induced histamine release from mast cells. The mechanism by which this occurs is not yet defined, but may include a decreased level of homocytotropic Ab. However, as with any system involving protein synthesis there appears to be a balance of repression and activation factors to control the anaphylactic
response. This may provide for gradations in response, as well as the phenomenon of 'breakthrough' reactivity, and the wide variation in reactivity among animals.
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