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The Regulation of Pulmonary Immunity

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I. Introduction

The human lung is exposed daily to over 10,000 liters of inspired, ambient air and the continuous aspiration of small amounts of nasopharyngeal secretions during sleep (Kikuchi *et al.*, 1994). Depending on the quality of air in the environment or the resident flora in the nasopharynx, the respiratory tree faces the enormous task of oxygenating blood across a moist, thin alveolar–capillary wall (approximately 1 μm) and yet resisting infection. Mechanical mechanisms and other innate host defenses are important in preventing infection, but acquired immunity is essential to prevent recurrent and chronic infection as made strikingly evident by the increased numbers and severity of pulmonary infections in immunocompromised hosts (Newhouse *et al.*, 1976; Mason and Nelson, 1992).

The lung, as is true of other epithelial surfaces that interface with the environment, has developed several strategies to avoid infection. As an important component of these strategies, the host must be able to downregulate both nonspecific and immune-mediated inflammation.

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Failure to regulate local immunity results in diseases such as asthma, hypersensitivity pneumonitis, and perhaps sarcoidosis and idiopathic interstitial pneumonitis (Holt, 1993; Djukanovic *et al.*, 1990; O'Connor and FitzGerald, 1992). Thus, the desire to enhance protective pulmonary immune responses by vaccination and to prevent or control unwanted responses underlie the need to study basic immune mechanisms in the lung. Furthermore, understanding critical immunoregulatory mechanisms may lead to strategies for preventing and controlling lung transplant rejection and immune-mediated lung damage in bone marrow transplant patients.

A number of recent reviews have discussed important issues in the development of pulmonary immune responses (Agostini *et al.*, 1993; Hance, 1993; Lipscomb *et al.*, 1993a; Gyetko and Toews, 1993; Holt, 1993; Bice, 1993). The goal of this chapter is to describe the cells and structures of the lung that participate in pulmonary immunity and to summarize studies that help explain how the lung responds to challenges with foreign antigens, with particular emphasis on animal models that have been developed to explore these issues. Features of the immune apparatus that are unique to the lung will be highlighted, and important questions currently under investigation will be indicated.

II. Immune Cells and Structures of the Lung

An important challenge is to understand how the host protects itself from infection yet regulates immunity to prevent tissue damage. Tissue culture has been a powerful tool for understanding how immunologically relevant cells interact. The study of how various extracellular signals influence gene expression in cultured cells has given important insight into how the milieu could influence cell behavior at various anatomic sites. However, because it is not yet possible to know what all of the influences within a tissue are, hypotheses generated from cells in culture must be tested *in vivo*. For example, alveolar macrophages (AM) exist attached to epithelial cells and migrate within a layer of surfactant rather than attached to plastic in a layer of medium. A recent review in this series discussed the importance of placing lymphocytes in their spatial context within the host to properly understand their function (Kroemer *et al.*, 1993). This consideration is especially important in the lung.

In considering the development of pulmonary immunity in a spatial context, it is useful to divide the evolution of an immune response in the lung into three distinct but overlapping phases (see Fig. 1; Lipscomb, 1993a): (1) in the *afferent phase,* antigen reaches the lung, is

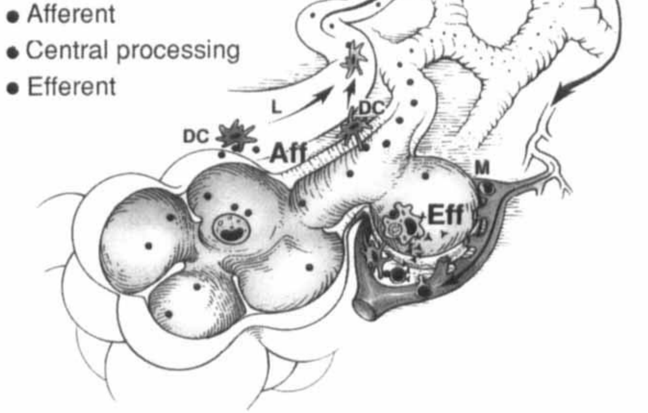

FIG. 1. A model for afferent, central processing, and efferent phases of a pulmonary immune response. In the afferent phase (Aff), antigen that reaches bronchoalveolar spaces can directly enter lymphatics (L) or be processed by intraepithelial or interstitial dendritic cells (DC) that enter lymphatics and migrate to lung-associated lymph nodes (LALN). In the central processing phase (CP) antigen on lung DCs (or, perhaps, antigen processed by resident lymph node DCs) present antigen to T lymphocytes (T) to initiate the expansion of T cell clones that, in turn, may help B cells expand when B cell immunoglobulin receptors recognize "native" antigenic determinants. In the efferent phase (Eff), recently activated T cells and B cells (not shown) leave LALNs and circulate; they are subsequently recruited to the lung at sites of inflammation. Exposure of T cells to relevant antigen results in the release of cytokines that amplify an inflammatory response by recruiting nonspecific effector cells, such as monocytes (M), to the site and activating them to kill and/or growth-inhibit microorganisms.

take up by antigen-presenting cells (APCs), and presented to naive T cells expressing the relevant T cell receptors (TCRs); (2) in the *central processing phase,* specific lymphocyte clones are expanded and differentiate; and (3) in the *effector phase,* effector T cells and B lymphoblasts find their way to pulmonary sites requiring expression of a specific immune response. At each phase, events must be tightly regulated to allow an effective immune response yet avoid excess, potentially destructive inflammation. The location in the respiratory tract where each of these phases occurs is somewhat controversial, but the bulk of evidence indicates that in the normal host after reaching the lungs, antigen is carried on APCs, in phagocytes, or free in lym-

phatic fluid to draining lung-associated lymph nodes (LALNs) (Lauweryns and Baert, 1976–1977; Lehnert, 1992) where central processing occurs. Effector cells are released into the efferent lymph and reach the blood stream where they are recruited from the vasculature into the lung (Berman *et al.*, 1990).

Many investigations have considered the lung in the broader context of mucosal immunity. This conceptual framework is useful, but the respiratory tract has several distinctive features that require that initiation and expression of lung immunity be considered separately from immune responses at other mucosal sites.

A. Concept of an Upper and Lower Respiratory Tract

The respiratory tract shares features of other organs, i.e., skin, gut, and urogenital tract in which an epithelial layer interacts with the environment. For internal organs, the concept of a common mucosal system was developed based on evidence that immune lymphocytes generated at one surface migrated to both homologous and distant mucosal sites (McDermott and Bienenstock, 1979; McGhee *et al.*, 1992). We will return to this concept shortly, but in addition to this concept, the lung must be understood immunologically from the point of view that both an upper and lower respiratory tract system exist (Kaltreider, 1976; Kazmierowski *et al.*, 1977), and each system exhibits distinctive as well as common immune mechanisms.

The upper respiratory tract starts at the nares and extends to the level of the terminal bronchioles. A pseudo stratified to single layered columnar epithelium covers vascularized connective tissue, the lamina propria, which, depending on the level of the airway, also contains variable numbers of mucous glands, smooth muscle, and, in the larger airways, is bounded by cartilage. By contrast, in the lower respiratory tract (which by definition includes the alveolar ducts and alveoli), the epithelium is markedly attenuated and frequently separated from the pulmonary capillary endothelium by only a fused basement membrane. The mechanisms of antigen handling and the types, location, and numbers of immunologically relevant cells differ even within areas of the upper respiratory tract, but are most strikingly different between the upper versus lower respiratory tracts. For example, mucociliary clearance is the major mechanism for clearance of particulates in the upper tract. In contrast, phagocytosis by resident AM which subsequently attain the level of the upper tract to be removed by the mucociliary elevator characterizes particulate clearance in the lower tract (Lauweryns and Baert, 1976–1977; Lehnert, 1992).

Another distinctive feature of the upper versus lower tracts relates

to the organization of lymphoid tissue. In the upper tract, lymphocytes reside in both aggregates and diffusely distributed along the mucosa of the upper tract, and in some animal species may infiltrate the epithelium (Bienenstock *et al.*, 1973a,b; Sminia *et al.*, 1989). In the lower tract, lymphocytes are present in variable numbers both within alveoli and in the interstitium, but, in the normal host, organized aggregates do not occur (Sminia *et al.*, 1989; Pabst, 1992).

Other important features of the upper versus lower tract involve the relative importance of IgA as the protective antibody. Thus, IgA-secreting B cells occur in the mucosa of the upper tract, and IgA is the major immunoglobulin in secretions of the upper respiratory tract; although IgA is present in the lower tract, IgG and IgM predominate in bronchoalveolar lavage (BAL) fluids (Kaltreider, 1976).

The development of immunity in the lung, as elsewhere, requires that relevant cells display appropriate surface molecules for contact and secrete appropriate factors. Some ligand–receptor interactions are specific while others are not, and it is the particular pattern of surface molecules and secreted factors expressed by interacting immune cells that determines the type of immune response that develops during central processing. Furthermore, at sites of pulmonary inflammation, other patterns of adhesion molecules and cytokine/chemokine expression by immune and parenchymal cells direct the tempo and magnitude of accumulation of recruited cells. Many recent studies emphasize the critical importance of cytokines and chemokines and the expression of adhesion molecules in regulating pulmonary inflammation and immunity (Kunkel *et al.*, 1989; Stein-Streilein and Phipps, 1993; Redington *et al.*, 1993; Standiford *et al.*, 1993; Jordana *et al.*, 1993; Lukacs *et al.*, 1994a).

The cells that are the major initiators and regulators of immunity in the lung include macrophages, dendritic cells (DCs), and lymphocytes, each expressing surface molecules and secretory products that depend on perturbations in the environments. However, other cells in the milieu, e.g., epithelial cells, fibroblasts, mast cells, and various recruited blood leukocytes, also play important regulatory roles which will only be briefly examined in subsequent sections. First, however, the special feature of lung macrophages, DCs, and lymphocytes are discussed.

B. Lung Macrophages

Originally proposed as important APCs in the lung (Lipscomb, 1988), this diverse group of cells is now best understood in the context of lung immunity as phagocytes and as regulators of both immunity

and nonspecific inflammation (Holt, 1986; Lipscomb *et al.*, 1993a). Indeed, the bulk of evidence indicates lung macrophages are unlikely APCs in the initiation of primary immune responses. These cells reside within the airways at all levels of the respiratory tract, in the lamina propria, the interstitium, the alveolar regions and pleura, and within pleural spaces; in ruminant species, lung macrophages are found within the pulmonary capillaries (Lehnert, 1992; Brain, 1992). All lung macrophages originate from the bone marrow (van Oud Alblas *et al.*, 1983; Springmeyer *et al.*, 1982; Godleski and Brain, 1972), but maintenance of at least some of these resident populations partly derives from self-replicating pools (Pinkett *et al.*, 1966; Bowden and Adamson, 1980; Sorokin *et al.*, 1984; Tarling *et al.*, 1987; Shellito *et al.*, 1987).

Pulmonary macrophages are both phenotypically and functionally diverse, even within a single compartment. Because of ready access to bronchoalveolar macrophages using BAL, the diversity of these cells has been most frequently studied. Macrophages obtained by lavage include resident AM as well as macrophages that reside within the lumen of the bronchi and bronchioles (intraluminal macrophages); a distinction between these two cell populations cannot be readily made. However, the vast majority of cells obtained during a human lavage are from the alveoli, since the technique is performed with a wedged flexible fiberoptic bronchoscope and the large alveolar surface area relative to the bronchial surface area is sampled (Reynolds, 1987; American Thoracic Society, 1990). In small rodents, e.g., mice and rats, a catheter is typically placed in the trachea and a larger proportion of the recovered cells are from the bronchi and bronchioles, but the majority of cells are still from alveoli if the procedure is performed correctly.

Functional attributes of subpopulations of resident AM have been studied. Most studies exploit the differences in density (Murphy and Herscowitz, 1984; Shellito and Kaltreider, 1985; Oghiso, 1987), predominantly a function of cell size. Another fractionation technique depends on differences in the cells' capacity to be readily lavaged, a function of their adherence to epithelium and/or their residence in the bronchial lumen versus in alveoli (Holt *et al.*, 1982). Within subpopulations, AM differ in expression of class II major histocompatibility antigens (MHC), Fc and complement receptors, phagocytic capacity, responses to chemotactic stimuli, cytotoxicity, cytokine production, and capacity to suppress *in vitro* immune responses (Holt *et al.*, 1982; Murphy and Herscowitz, 1984; Shellito *et al.*, 1983; Shellito and Kaltreider, 1985; Oghiso, 1987). Furthermore, the size, function, and phenotype of AM shifts during an inflammatory response (van Oud Alblas

et al., 1983). Although alternate explanations for these changes have been offered, the most likely one is that AM recently arrived from the peripheral blood are similar in the size and phenotype to circulating monocytes, the precursors of AM. Thus, in the study of AM and their subpopulations and, indeed, in any study of lung immunity, it has become a dictum that investigators must be careful to avoid low levels of chronic inflammation by housing experimental animals in specific pathogen-free environments. Because respiratory infections are so common in nearly all animal species and can markedly affect experimental results, this consideration cannot be overstated.

Interstitial macrophages (IM) when compared with AM also exhibit size, functional, and phenotypic differences. These differences may reflect the stage of differentiation from blood precursors, but more likely reflect the environment and physiological roles of phagocytes in these two distinct locations. Important membrane molecules that determine the function of macrophages include complement, Fc, mannose, and scavenger receptors, as well as class I and II MHC, adhesion, and other signaling molecules. Important differences in many of these exist depending on the location of lung macrophages. For example, in mice, IM express C3 receptors, whereas most resident AM do not (van Oud Alblas and Van Furth, 1979).

Few studies exist on the role of interstitial, pleural, or intravascular lung macrophages in immune responses. However, numerous studies have examined the role of AM in stimulating mitogen, alloantigen, and antigen stimulated responses *in vitro*. Not surprisingly, these studies have often reached conflicting conclusions, a result of the differing animal species used, the level of superimposed AM suppressive activity, and the assay procedure that was used (Holt, 1986; Lipscomb, 1988). Most studies support the concept that AM are poor APC for priming T cells even when they express high levels of class II MHC, as human AM do (Holt, 1979; Ansfield *et al.*, 1979; Toews *et al.*, 1984a; Lipscomb *et al.*, 1986). Bronchoalveolar cells, which contain up to 90% AM, fail to act as effective APC *in vitro* because they are either actively suppressive or because they fail to express some other poorly understood accessory function which may manifest as poor lymphocyte-accessory cell binding (Shellito *et al.*, 1983; Lyons *et al.*, 1986; Kradin *et al.*, 1987). Most current evidence indicates that DCs are the most efficient APC in stimulating naive T cells, especially CD4 T cells, although class II MHC-positive cells of many cell types are capable of stimulating recently primed T cells (Steinman, 1991; Croft, 1994). Variable contamination of cells with lung DCs (Pollard and Lipscomb, 1990) and the presence of recently activated T cells in

responder populations may well explain reports that AM can function as effective APC (Rich *et al.*, 1987).

Mechanisms utilized by populations of BAL cells to suppress immune responses are likely to depend on variables of the assay systems and are particularly dependent on animal species. In dogs, PGE_2 production (Demenkoff *et al.*, 1980) and the synergistic activity of PGE_2 and oxygen radicals (Kaltreider *et al.*, 1986) were shown to be important. In humans, AM/lymphocyte contact leading to inhibition of receptor-induced intracellular calcium increases in responder T cells has been described (Yarbrough *et al.*, 1991). Resident murine macrophages, particularly at high numbers relative to numbers of stimulator lung DC, suppress a mixed lymphocyte reaction (MLR) by secreting TGFβ (Lipscomb *et al.*, 1993b). Furthermore, nitric oxide (NO) made by murine AM may inhibit the development of potent APC cell function of lung DC (Holt *et al.*, 1993). Evidence that AM suppression plays a role *in vivo* has been indirectly shown by depleting AM with an intratracheal dose of liposomes containing a macrophage cytotoxic drug (dichloromethylenediphosphonate) followed by immunizing via the respiratory tract. The animals demonstrated increased numbers of antibody-forming cells (AFCs) in LALNs compared to immunized controls pretreated with only liposomes (Thepen *et al.*, 1989). IgG, IgA, and IgE AFCs were all increased.

AM-suppressive activity is important to prevent the development of hypersensitivity reactions, but in circumstances in which lung immunity is important for protection, AM-suppressive activity could be counterproductive. However, suppressive activity of murine macrophages can be inhibited by exposure to GM-CSF and to a lesser extent by other selected cytokines (Bilyk and Holt, 1993). The major role of AMs seems to be to phagocytose and remove potentially dangerous particulates and soluble antigens from the alveoli and to inhibit local lung immune responses. However, immunity may develop in the presence of otherwise suppressive AMs by the recruitment of leukocytes to the alveolus with opposing activity or by strong environmental influences that result in cytokine secretion that diminishes AM suppressor function.

C. Lung Dendritic Cells

Immunologists have long recognized that adherent cells are required for optimal *in vitro* immune responses whether one measures T cell lymphoproliferative responses, T cell cytokine production, or T-dependent B cell responses. They have also developed an increasing appreciation of the role of DCs in priming naive T cells since they

were first identified in the spleen (Steinman and Cohn, 1973). Since then, the role of DCs in immune responses initiated at the epithelial surface is being clarified. Little doubt exists that DCs play a pivotal role in initiating immune responses in the skin (reviewed by Steinman, 1991). Furthermore, substantial support has developed for the concept that intraepithelial DCs are functionally different from those that have migrated into regional lymph nodes. Thus, freshly isolated Langerhans cells are capable of processing antigen and stimulating T cell clones, but they require "maturation" *in vitro* before they are fully effective in stimulating naive T cells in an MLR; at this time they have a markedly reduced capacity to process antigen (Romani *et al.*, 1989). Data indicate that lung DCs share a number of characteristics of skin DCs and likely also play a critical role in initiating lung immunity (Holt, 1993).

APCs must not only process antigen and express it in the context of class II MHC, but also express appropriate accessory molecules to enhance the interaction of the APCs with T cells. Recent studies emphasize the importance of the interaction of B7 on the APC cell surface with CD28 and/or CTLA4 expressed on responder T cells to deliver a second signal (reviewed by June *et al.*, 1994). Failure to trigger this second signal may cause T cells interacting with APCs via only the TCR–peptide/MHC II interface to become anergic (Harding *et al.*, 1992; Boussiotis *et al.*, 1993; Chen and Nabavi, 1994) and produce a state of tolerance *in vivo* (Van Gool *et al.*, 1994). B7 is a receptor family of at least two molecules in the immunoglobulin supergene family. B7-1, (CD80), and B7-2 (CD86) are both expressed constituitively on DCs in contrast to macrophages and B cells that must be activated to express these molecules (Vandenberghe *et al.*, 1993). Other important accessory molecules, usually identified phenotypically by standard immunocytochemistry and functionally by the ability of specific antibodies to block an immune function, include CD40, CD54 (ICAM-1), and CD58 (LFA-3) (Steinman, 1991).

1. *Isolation and Characterization*

Like DC from other sites, lung DC constituitively express both class I and II MHC, are light density, loosely adherent, poorly or nonphagocytic, and demonstrate long dendritic processes both in tissue sections and in cell suspensions (Holt *et al.*, 1985;1988; Sertl *et al.*, 1986; Rochester *et al.*, 1988; Nicod *et al.*, 1987; Pollard and Lipscomb, 1990). Lung DCs fail to express pan T, natural killer (NK) cell, B cell and *many* macrophage markers. The critical accessory molecules CD54 and CD58 (Xia *et al.*, 1991; Nicod and El Habre, 1992) are

present on many lung DCs, but data are incomplete on the expression of CD40, CD80, and CD86 family.

The high levels of constitutive class II MHC and the dendritic shape of DCs has been exploited to examine their location in tissue sections of lung by a number of investigators. DCs form an interdigitating network in the airway epithelium of all species in whom they have been investigated (Sertl *et al.*, 1986; Holt, 1993) similar to the network described for skin Langerhans cells. Intraepithelial DCs are particularly dense in the trachea and gradually diminish in concentration as the airways branch, but are increased at sites of chronic inflammation (Schon-Hegrad *et al.*, 1991). DCs also exist in the connective tissue surrounding bronchi and bronchioles, in perivascular connective tissue, in alveolar septa, in the pleura, and in very small numbers in alveolar spaces (Sertl *et al.*, 1986; Holt and Schon-Hegrad, 1987; Kradin *et al.*, 1991; Havenith *et al.*, 1992; van Haarst *et al.*, 1994). In rats, intraperitoneal injections of IFNγ increased the numbers of intraepithelial and septal DCs without increasing their accessory cell function (Kradin *et al.*, 1991), and inoculation of Bacillus Calmette–Guerin (BCG) increased the numbers of DC that could be lavaged from the alveolus (Havenith *et al.*, 1992). Thus, the numbers of lung DCs in various anatomic sites depend on signals delivered that are coincident with inflammation.

Lung DCs have been isolated with a variable degree of success utilizing adherence, density, and class II MHC expression properties; their function and other phenotypic features have been studied. Lung macrophages are the most difficult cells to separate from DCs in single cell suspensions of lung cells. Effective procedures for isolating fairly pure DC populations require exploiting the phagocytic and autofluorescent properties of lung macrophages (Nicod *et al.*, 1987,1989a; Pollard and Lipscomb, 1990; Havenith *et al.*, 1993a). Using these techniques, the function of DC-enriched lung cells has been assessed. Functional assays have included stimulation of periodate-treated lymphocytes, MLRs, and antigen-induced stimulation of either memory T cells or lymphoblasts. All have shown that lung DCs function as well or better than DCs from other sources (Nicod *et al.*, 1987; Rochester *et al.*, 1988; Pollard and Lipscomb, 1990).

2. *Phenotypic Heterogeneity*

DCs isolated from whole lung preparations are phenotypically heterogeneous, and cell markers differ somewhat among animal species and even within individuals of a species. An example of this latter variation relates to CD1a (OKT6), expressed by human Langerhans

cells in the skin, which has been variously described as being present on from less than 1% (Sertl *et al.*, 1986; Nicod *et al.*, 1987) to 30% (van Haarst *et al.*, 1994) of lung DCs in man. This antigen may be important in stimulating $\gamma\delta$ T cells, a potentially important interaction for host defenses in the lung in view of observations that $\gamma\delta$ T cells may recognize heat-shock proteins of *Mycobacterium tuberculosis* (Mtb) (Born *et al.*, 1991, Kaufmann and Kabelitz, 1991).

Variation in expression of cytoplasmic and surface markers also occurs *within* populations of lung DCs. For example, in the mouse, the interdigitating cell antigen (NLDC-145) and CR3 are present on about half of lung DCs, while the majority of lung DCs express CD25 and the heat-stable antigen (as defined by J11D; Pollard and Lipscomb, 1990). The latter two markers are also uniformly expressed on murine thymus and skin DCs, but are absent from the majority of splenic DCs (Crowley *et al.*, 1989), indicating the likelihood of a closer relationship of lung DCs to tissue DCs rather than to DCs that primarily home from bone marrow to lymphoid tissue. Additional evidence that lung DCs are distinct from the majority of splenic DCs is that lung DCs fail to express the splenic DC marker recognized by the monoclonal antibody 33D1 (Pollard and Lipscomb, 1990).

In both rat and mouse, another heterogeneous marker is FcRII, positive on about half of lung DCs (Pollard and Lipscomb, 1990; Xia *et al.*, 1991). Interestingly, Langerhans cells freshly isolated from the skin express FcRII which is downregulated as the cells mature in culture. Thus, it is possible that the FcR expression in the lung denotes a population similar to freshly isolated skin DCs. In the rat, DCs in the epithelium lining the airways are more likely to be FcR^+, whereas parenchymal DCs are nearly uniformly FcR^- (Gong *et al.*, 1992). Murine DC in the epithelium of the trachea also express FcRII (Sertl *et al.*, 1986). This suggests the possibility that intraepithelial DCs are poised to take up and process antigens at the epithelial/environment interface and enter the interstitium to traffic to LALN. Thus, at least some interstitial DCs which fail to express FcR may be in transit. On the other hand, the presence of FcR^- DCs in lung parenchyma, including alveolar septa, may indicate distinct immune functions for this subset of lung DCs.

No difference was found in mice in the capacity of the FcR^+ or FcR^- subsets to stimulate an MLR (Pollard and Lipscomb, 1990). However, in the rat, the FcR^- population was a more potent stimulator of naive T cells in an MLR and in responses to lectins, but FcR^- and FcR^+ were equally capable of presenting soluble and particulate antigens to antigen-sensitized T cells (Kradin *et al.*, 1993). Of interest

in these later studies was that 70% of FcR^- and only 39% of FcR^+ cells expressed the adhesion molecule CD54 (Kradin *et al.*, 1993), an observation that might partly explain the increased ability of the FcR^- cells to stimulate an MLR. In elegant studies in which lung intraepithelial DCs were isolated from the rat, these cells were shown to present antigen to primed T cells more efficiently than did parenchymal DCs. In man, further enrichment for FcR^+ decreased the ability of lung cells enriched in DC to stimulate an MLR (Nicod *et al.*, 1987), although these studies are complicated by the large numbers of contaminating FcR^+ macrophages in the DC populations. Nevertheless, FcR positivity has been a useful marker for identifying subsets of lung DCs and the majority of data are consistent with the concept derived from Langerhans cells that a population of FcR^+ intraepithelial DCs may take up antigen from the bronchial lumen and differentiate into cells that can stimulate naive T cells upon migration into draining lymph nodes.

3. *Origin*

DCs in all tissue sites originate from the bone marrow (Steinman, 1991). They can be cultured from precursors in bone marrow and peripheral blood using GM-CSF and, for adult human DCs, from peripheral blood with IL4 (Inaba *et al.*, 1992,1993; Thomas *et al.*, 1993; Sallusto and Lanzavecchia, 1994). In one study, Ia^+ lung DCs were first recognized in rat lung parenchyma in fetal life at Day 15 of gestation (McCarthy *et al.*, 1992). Intraepithelial DCs were present by Day 17 and continued to increase during postnatal development (McCarthy *et al.*, 1992). Comparison of fetal lung DCs demonstrated that they were not as efficient as adult DCs in stimulating an immune response, but were fully functional at birth. In contrast, another study failed to detect Ia^+ DCs in rat lung epithelium or parenchyma until birth, and the numbers increased rapidly until 3 weeks of age when they approximated those found in adults (Nelson *et al.*, 1994). Ia^+ DCs were first observed in the nasal turbinates and intensity of Ia staining increased in time, first in the trachea and later in lung parenchyma, compatible with environmental exposure effecting the change. Using another marker for DC, OX62 (which also detects $\gamma\delta$ T cells), these same authors found CD3-negative, OX62-positive DCs at all levels of the lung in fetal rat lung and speculated that environmental influences upregulated Ia expression and subsequent function of these cells. Consistent with this speculation was that $IFN\gamma$ increased the numbers of Ia^+ cells in airway epithelium while steroid inhalation decreased the numbers relative to control rats. The conflicting findings of the

two groups may be based on the differences in the housing environments for the two groups of rats. Thus, Nelson and collaborators (1994) used dust-free bedding in contrast to McCarthy *et al.* (1992). It was quite likely that *in utero* influences affected the numbers and function of lung DCs.

4. *In Vivo Function*

Numerous studies have demonstrated that DCs from spleen, lymph node, and skin have extraordinarily potent ability to immunize recipient animals following inoculation either iv or subcutaneously (Knight *et al.*, 1983; McKinney and Streilein, 1989; Sornasse *et al.*, 1992). Lung DCs have not been used to immunize experimental animals to date, but several lines of evidence suggest that they have an important role *in vivo*. As discussed, their location within the epithelium places them in an optimal position to take up and process antigens that breach the epithelial barrier; perivascular and septal DCs should be poised to process antigens that reach the lung via the vasculature. Studies have shown that splenic DCs instilled into the lung may reach LALN (Havenith *et al.*, 1993b) and if pulsed with antigen may induce an immune response (Havenith *et al.*, 1993c). In this latter study, AMs pulsed with antigen were also capable of initiating an immune response, but in contrast to DCs heat-killed AMs were also able to immunize suggesting that AMs stimulated responses by having the antigen reprocessed by the host's own APCs. Additionally, explanted lung DCs had gained the ability to stimulate primed T cells following the intratracheal delivery of the relevant antigens (Holt *et al.*, 1993). Last, consistent with the dynamic activity of epithelial DCs in carrying environmental antigens into LALN, irradiation of rats resulted in a loss of 85% of resident DCs by 72 hr and reconstitution of tracheal DCs from bone marrow precursors by 10 days (Holt *et al.*, 1994). Lung DCs most surely play a major role in regulating lung immunity and, as discussed previously, are likely themselves regulated by multiple environmental factors. It remains to be determine whether antigen presented by lung DCs is more likely to result in TH1 versus Th2 responses in the lungs and whether bypass of DCs in immunization of the host is more likely to deliver a tolerogenic signal, or whether other factors are more important in determining these outcomes.

D. Lung Lymphocytes

T cells, B cells, and NK cells have all be described in various lung compartments (Holt and Schon-Hegrad, 1987; Stein-Streilein, 1988; Pabst, 1990; Agostini *et al.*, 1993). Their phenotype and function have

been described either by evaluating markers *in situ* or by obtaining cells from BAL or collagenase digestion of lung tissue. As with lung macrophages and DCs, lung lymphocytes are a dynamic population with the capacity to enter and leave the lung depending on influences in the milieu.

1. *Location*

In several species including rat, rabbit, and chicken, large numbers of lymphocytes are located in organized bronchus-associated lymphoid tissue (BALT), which is discussed below. However, BALT is not constitutive in all species and not regularly seen in hamsters, mice, and humans. In these latter species, the majority of lung lymphocytes are present in the interstitium, diffusely scattered in the mucosa, alveolar septa, or pleura. Also, depending on the species, there may be an intraepithelial and/or an intravascular pool of lymphocytes (Pabst, 1990). A population of lung lymphocytes exists in the bronchoalveolar spaces and is recovered by BAL. In animals kept in specific pathogen-free environments, lymphocytes range from 5 to 10% of cells recovered by BAL (Agostini *et al.*, 1993; Pabst, 1990). In normal, nonsmoking humans the figures vary from less than 8 to 20% (Daniele *et al.*, 1975; Davidson *et al.*, 1985; Becker *et al.*, 1990).

The proportion of T cells to B cells and NK cells varies to some degree with the compartment in which the lymphocytes are present. The composition of lymphocytes in BAL fluids is generally representative of the proportion of T cells, including CD4 and CD8 T cells, and B cells found in peripheral blood. Interestingly, in humans, differences in the phenotype and function of NK cells occur between the peripheral blood and the alveolus. Thus, NK cells in human alveoli fail to express cytolytic activity and are largely negative for CD16, although CD16-expressng NK cells with cytolytic activity exist in the interstitium (Weissler *et al.*, 1987). Relatively high numbers of cytolytically active NK cells also exist in the interstitium of mice. Fifteen to 20% of cells fractionated on nylon wool columns following isolation from enzyme-digested lungs express the allotypic NK1.1 marker, detectable in C57BL/6 mice (Stein-Streilein *et al.*, 1983).

When leukocytes are isolated from the lung parenchyma of guinea pigs, mice, rats, and humans, the relative percentage of lymphocytes varies from 15 to over 50%. The variance likely depends on the strain of animal, whether they are kept specifically pathogen-free, and the rigor by which small monocytes are excluded (much of the data are derived from examining Wright–Giemsa-stained cytospin preparations) (Lipscomb *et al.*, 1982; Stein-Streilein *et al.*, 1983; Holt and

Schon-Hegrad, 1987; Nicod *et al.*, 1989a,b). Flow cytometric analysis of T cell and B cell populations of lung lymphocytes indicates that the relative proportion of B cells is either increased or the same, and the CD4/CD8 ratios are either decreased or similar compared to peripheral blood (Holt *et al.*, 1986; Abraham *et al.*, 1990; Marathias *et al.*, 1991; Huffnagle *et al.*, 1994).

Intraepithelial lymphocytes are common in the gut mucosa and exist in lung epithelium of some animal species, but are relatively less common than those in the gut (Holt and Schon-Hegrad, 1987; Fournier *et al.*, 1989). In humans, no B cells were found in airway epithelium, and CD8 outnumbered CD4 T cells (Fournier *et al.*, 1989). In an examination of epithelium of the upper respiratory tract (in the nose and covering the tonsil and adenoids), both B cells and T cells were found. Notably, $\gamma\delta$ T cells occurred in aggregates where they comprised up to 30% of the total T cells, although in general 80–90% of T cells expressed the $\alpha\beta$ TCR (Graeme-Cook *et al.*, 1993).

In studies examining cells isolated from human lung parenchyma, $\gamma\delta$ T cells made up less than 5% of T cells and were $CD3^+$, $CD4^-$, and $CD8^-$ (Abraham *et al.*, 1990; Marathias *et al.*, 1991). In a study of mice, on the other hand, 8–20% of resident lung lymphocytes were $CD3^+$ and $\alpha\beta$ TCR^-, and were presumably $\gamma\delta$ T cells. $\gamma\delta$ T cells increased following aerosol delivery of *M. tuberculosis* (Augustin *et al.*, 1989).

2. *Traffic*

The regulation of movement of lymphocytes into and out of the lung is still incompletely understood. Nevertheless, with increased information about the role of adhesion molecules in regulating the traffic of lymphocytes (Springer, 1994), investigators are beginning to unravel what regulates steady-state movement of cells into the lung as well as possible preemptive signals that occur with inflammation. Research on emigration of cells into the lung has centered on the accumulation of neutrophils within pulmomary vasculature and their immigration into the air spaces of the lung because these cells are likely to play a critical role in the development of the acute respiratory distress syndrome. Recent information indicates that the adhesion molecules, P- and E-selectin, the integrins, and ICAM-1 and ICAM-2 play important roles in the accumulation of neutrophils in the lung (Pilewski and Albelda, 1993), The relevance of each adhesion molecule likely depends on the inflammatory stimulus that provokes the recruitment (Hellewell *et al.*, 1994; Doerschuk *et al.*, 1990; Doerschuk, 1992; Mulligan *et al.*, 1993a–e).

The regulation of lymphocytes into the lungs is less well understood even though, as mentioned above, evidence exists that lymphocytes isolated from LALN or collected from the efferent lymphatics of LALN have a predisposition to return to the lungs (McDermott and Bienenstock, 1979; Spencer and Hall, 1984; Joel and Chanana, 1987). Several studies have demonstrated that T lymphocytes isolated from lung lavages of normal humans predominantly express a memory phenotype, e.g., they are $CD45RO^+$, $CD45RA^-$ cells (Saltini *et al.*, 1990; Becker *et al.*, 1990). Other important markers for naive versus memory T cells are the hyaluronic acid receptor (CD44) and L-selectin. Naive T cells express low levels of CD44 and high levels of L-selectin, memory T cells express high levels of CD44 and low levels of L-selectin (reviewed in Sprent, 1994). Thus, with the recognition that memory T cells accumulate preferentially in the lung, the issue becomes what is the stimulus for their entry and why do naive cells fail to accumulate.

The role of naive T cells with their multitude of diverse receptors is to continuously recirculate through secondary lymphoid organs, i.e., spleen and lymph nodes, so that antigen-bearing APCs can interact with appropriate T cells at that site. This APC–T cell interaction results in clonal expansion and subsequently provides challenged tissues with a population of cells able to specifically react and protect the host. Thus, naive T cells have receptors that allow them to migrate to secondary lymphoid organs (Butcher *et al.*, 1990; Jutila 1994). In contrast, lymphocytes that must enter challenged or inflamed tissues might be expected to express a different set of homing receptors. Indeed, this is the case. Although accumulation in markedly inflamed sites seems to be nonspecific, when only low levels of inflammation exist, where immune cells accumulate may be determined in the draining lymph nodes from which they derive (Picker, 1994). Teleologically, this would be a more efficient way for the immune system to selectively redirect cells to sites where they are needed. Indeed, evidence suggests memory lymphocytes bearing the cutaneous lymphocyte-associated antigen seem to specifically home to the skin (reviewed in Picker, 1994). Studies also suggest that memory cells in the lung may have a unique set of homing receptors compared to memory cells in the skin or at mucosal sites (Picker *et al.*, 1994). Thus, $CD3^+$, CD45RO high/CD45RA low T cells in the skin were E-selectin$^+$ and CLA^+, but were $\alpha4\beta7^-$ and $\alpha_e\beta7-$, while lung memory T cells were E-selectin$^-$, CLA^-, and $\alpha4\beta7^-$, but 50% of the cells were $\alpha_e\beta7^-$. The lung phenotype was different from the overall memory T cell phenotype in blood, suggesting that there might be an unidentified receptor on lung T cells that specifically selected them for emigration into the lung.

An important issue is whether any naive lymphocytes traffic through the lungs. Naive T cells migrate into peripheral lymph nodes via high endothelial venules (HEV) using an L-selectin/peripheral lymph node addressin interaction. Some animal species have BALT in which HEV are present. Thus, in these animals, naive cells could enter the lungs and be available for primary immune responses to develop at these sites. However, evidence to support this possibility does not exist. Despite scanty information about the migration of naive and long-term memory T and B cells into the lungs, several studies have noted that lymphoblasts (or recently divided lymphocytes) have a tendency to migrate into both inflamed and uninflamed lung (Daniele *et al.*, 1977; Berman *et al.*, 1990). The location and mechanisms of lymphocyte transmigration are under investigation.

E. Organized BALT

Macrophages, DCs, and lymphocytes are diffusely distributed throughout all of the compartments of the lung. In addition, in most animal species examined, at least some organized lymphoid tissue may be found in variable amounts lining bronchi and bronchioles. These structures have been recognized for many years, but were first carefully described in 1973 (Bienenstock *et al.*, 1973a,b). More recently, some controversy about these structures has been raised because of the inability to readily detect them in all species. Thus, they are relatively rare in normal humans, cats, and young pigs (Pabst, 1990,1992). When first described they were compared to intestinal Peyers patches; they had the appearance of a follicle without a capsule. Furthermore, lymphocytes infiltrates the overlying bronchial epithelium which demonstrated alterations in morphology compared to adjacent epithelial cells. They were originally described in the bronchial mucosa of all species examined, e.g., rabbits, guinea pigs, rats, mice, dogs, pigs, chickens, and man (Bienenstock *et al.*, 1973a). These early studies revealed that neonatal thymectomy failed to affect the normal development of BALT in rats and chicks, and that tritiated thymidine labeling and autoradiography indicated that there was rapid cell proliferation of cells constituting BALT (Bienenstock *et al.*, 1973b). Transplantation of fetal lungs into extrapulmonary sites did not interrupt the development of the BALT, although it was not as cellular, suggesting that antigenic stimulation was required for full development.

Since these early studies, two comprehensive reviews have summarized the morphology and function of BALT (McDermott *et al.*, 1982; Sminia *et al.*, 1989). An individual aggregate of BALT or bronchus-associated lymphoid unit (BALU), a term defined by Sminia and col-

leagues, consists of a focal area of T and B cells admixed with fibroblasts, reticulum cells, macrophages, interdigitating cells which are comparable to Ia-positive DCs, and follicular dendritic cells. BALUs have no capsule, subcapsular sinuses, nor afferent lymphatics. Nevertheless, they have peripheral sinus-like lymphatics which subsequently drain into lymph nodes (Lauweryns and Baert, 1976–1977). Furthermore, there are arterioles, capillaries, and venules which include high endothelical venules (HEV) (Otsuki *et al.*, 1989). The structure of a BALU with the demonstration of HEV indicates the likelihood that naive T cells might migrate to these structures and initiate a primary immune response against an antigen translocated from the bronchial lumen, comparable to the role for Peyers Patches in the gut. Although early studies failed to demonstrate antigen could cross the overlying epithelium, more recent studies have suggested that soluble antigens might be translocated from the bronchial lumen across the epithelium overlying a BALU (Fournier *et al.*, 1977; Myrvik and Ockers, 1982; van der Brugge-Gamelkoorn *et al.*, 1985).

In BALUs lymphocytes are partitioned into B and T cell areas with central B cells surrounded by T cells. The dome over these areas and underneath the epithelium is a mixture of B and T cells (Sminia *et al.*, 1989). In BALUs, IgM- and IgG-bearing cells are present in significant numbers together with IgA-positive cells which contrasts with Peyers Patches in which IgA cells predominate (Sminia *et al.*, 1989). In rats, BALT has been examined with monoclonal antibodies to determine the type of T cells present; CD4-positive cells outnumber CD8-positive cells, but in the B cells aggregates nearly all of the T cells are CD4-positive cells (Sminia *et al.*, 1989).

Important studies relating to the traffic of T and B cells to BALUs have been published (van der Brugge-Gamelkoorn and Kraal, 1985) demonstrating *in vitro* binding of equal numbers of T and B lymphocytes to both rat and guinea pig BALUs. This finding is clearly different from Peyers Patch binding of T and B cells in which the numbers of B cells that bind are much greater than T cells that bind at a ratio of five B cells for every T cell (Stevens *et al.*, 1982). This finding corresponds to the increase in B cells in Peyers patches relative to the numbers of B cells in BALUs (Crawford and Miller, 1984). Taken together, these studies indicate that the specificity of HEV in BALUs is different from that in Peyers patches and more closely resembles the specificity of the HEV in mesenteric lymph nodes (van der Brugge-Gamelkoorn and Kraal, 1985).

It is important to address the issue of a similarity of BALT with gut-associated lymphoid tissue. BALT is prominent in certain species

including chicken, rabbit, and rat, but is clearly much less prominent in other species, including man. In some members of these species, BALT may be completely absent (Pabst, 1990). Nevertheless, even in man, other studies have shown that after birth, there is a gradual increase in loose aggregates of lymphoid cells that collect beneath the epithelium, particularly at points of bifurcation of bronchi. In man, mouse, and hamsters, these collections of lymphoid cells do not involve the epithelium nor modify the epithelium over the aggregate. In view of the relative lack of prominence of these structures in certain species, it suggests that their role in protective immune responses in the lung is not essential. Since an important function in the gut is for these structures to initiate IgA responses, it is possible that the lack of well-organized BALT in some species predicts that local initiation of IgA responses is not required for health. It also suggests that the common mucosal system in which IgA B cell precursors are developed in the gut and migrate to the lung may function to successfully protect the host from lung infections.

III. Lung Immunity to Noninfectious Particulate and Soluble Antigens

Since the late 1960s, investigators have been systematically exploring mechanisms in the development of immune responses to particulate and soluble protein antigens in the lung (Pepys, 1969). A major impetus was to understand what caused hypersensitivity lung diseases, such as asthma and hypersensitivity pneumonitis, to develop in some individuals, but not in others, although antigenic exposures were the same. Models to examine immune responses to various respiratory antigens were developed in many animal strains, including mice, rats, hamsters, guinea pigs, ferrets, dogs, monkeys, horses, and cattle; antigens were delivered via aerosol, intranasal, intratracheal, or intrabronchial instillation. The end point for immunity in experimental animals was generally measured by assessing the development of hypersensitivity disease clinically and morphologically, measuring serum and/or bronchoalveolar antibody, or characterizing some aspect of cell-mediated immunity (CMI) such as migration inhibition, delayed-type hypersensitivity (DTH) via skin test, or lymphoproliferation (Richerson, 1972; Newhouse *et al.*, 1976; Kaltreider, 1976; Kazmierowski *et al.*, 1977; Ganguly and Waldman, 1977).

Among the important findings of these early studies were that soluble antigens instilled into the lungs, in contrast to particulate antigens, often failed to produce immunologic lung damage (Schatz *et al.*, 1977; Fink, 1988), and that soluble antigen repeatedly instilled into the lung

could lead to local tolerance (Ratzjczak *et al.*, 1980; Holt and Leivers, 1982). Interpretation of early studies did not benefit from the current perspective that the type of immune response that develops, i.e., CMI versus antibody (including the predominant isotype of the antibody), is regulated by cytokines secreted by T cells and other cells present at sites of antigen deposition (Mosmann and Coffman, 1989). More recent studies have expanded the important concepts derived from earlier studies by focusing on the regulatory mechanisms in the development of lung immunity.

In order to understand how various forms of antigen might reach the immune apparatus and, therefore, antigen clearance is discussed. Then, while T and B cell immunity are clearly interdependent, studies that have focused on measuring specific T cell responses versus immunoglobulin synthesis in response to noninfectious lung antigens are summarized. Models that specifically examine the lung's response to infectious agents and alloantigens or lead to hypersensitivity disease are covered later.

A. Lung Clearance of Antigens

Many studies have shown that the LALNs are responsible for primary immune responses after lung immunization (Bice *et al.*, 1980b; Kaltreider *et al.*, 1983; Stein-Streilein *et al.*, 1979; Stein-Streilein and Hart, 1980; Lipscomb *et al.*, 1982). LALNs function as effective filters to remove particulate materials cleared from the lower respiratory tract via the lymphatics (Brain *et al.*, 1978; Green *et al.*, 1977; Morrow, 1972). Although LALNs are largely responsible for the induction of immunity after primary immunization, the mechanisms responsible for the clearance of antigen from the lung to LALNs are not completely understood. Most antigen deposited in the lung is cleared by phagocytosis by AMs and neutrophils that transport foreign material up the mucociliary escalator and out of the lung, although some antigen is transported to LALNs where an immune response is produced. At least some of this latter antigen is carried free in lymphatic fluid and apparently occurs in circumstances of limited inflammation (Lauweryns and Baert, 1976–1977).

The induction of pulmonary inflammation by antigen exposure appears particularly important in the translocation of immunogens from the lung to LALNs. Exposure of the lung to noninflammatory doses of antigen often fails to induce immune responses (Yoshizawa *et al.*, 1982; Bice *et al.*, 1991). It is possible that an immune response to airway antigens requires a dose that overwhelms normal phagocytic and clearance mechanisms (Bice and Muggenburg, 1988; Bice *et al.*, 1991). The observation that elevated immune responses are produced

in LALNs if antigen is deposited in the lungs of animals that have inhaled inflammagens further supports the importance of pulmonary inflammation in the translocation of antigen from the lung to the LALNs (Bice *et al.*, 1985,1987b). It is possible that inflammation may also alter the relative proportion of antigen reaching LALNs in cells as opposed to antigen free in lymphatic fluid.

In dogs inoculated via the airways with sheep red cells, a large number of neutrophils enter the lung from the vasculature with a peak response about 1 day after instillation of antigen (Bice *et al.*, 1989). Furthermore, neutrophils can phagocytize particles in the alveoli and migrate to the LALNs carrying the particles (Harmsen *et al.*, 1987). AMs can also phagocytize particles in the lung and transport them to LALNs (Corry *et al.*, 1984; Harmsen *et al.*, 1985). The relative contribution of neutrophils and AM in the translocation of antigen from the lung to LALNs is not known. However, neutrophils with phagocytized particles reach LALNs earlier than AMs and, may be more important for antigen transport to LALNs than AMs. Antigen transported to the LALNs may be released from both neutrophils and AMs and reprocessed by resident APCs to initiate pulmonary immunity. In addition to AMs and neutrophils, as previously discussed, lung DCs also likely carry antigen to the LALNs, and recent studies in which pulsed splenic DCs inoculated into the trachea were capable of immunizing the hosts supports this concept (Havenith *et al.*, 1993c). Whether antigen or lung DC initiates a different type of T helper subset response than antigen arriving in phagocytes or free in lymph has not been determined. Nevertheless, if lung inflammation enhances the transport of antigens from the lung to the LALNs, it is possible that inhalation of materials that induce pulmonary inflammation might lead to increased recognition of airborne antigens. Thus, pulmonary inflammation caused by inhaled pollutants (Osebold *et al.*, 1980) and passive cigarette smoke (Murray and Morrison, 1988; Ehrlich *et al.*, 1992) might increase the immune recognition of allergens and be responsible for increasing rates of asthma (Evans *et al.*, 1987; Platts-Mills *et al.*, 1991). In addition, inflammation induced by pulmonary viral infections may also be important in the induction of immunity to low levels of environmental antigens, e.g., allergens responsible for asthma (Castleman *et al.*, 1990; Duff *et al.*, 1993).

B. T Cell-Mediated Lung Immunity

Several important questions relate to the development of CMI in the lung and include (1) do antigens instilled into the lung cause local and/or systemic CMI? (2) Does the form of antigen, i.e., soluble, particulate, expressed by viable microorganisms influence the out-

come? (3) Does iv or subcutaneous inoculation of similar antigens generate similar degrees of CMI in the lung? (4) What redirects the immune T cells back into the lungs?

Inoculation of antigens into the lung can result in both local and systemic CMI (Kaltreider, 1976). As discussed previously, both soluble and particulate antigens can induce immunity, but soluble antigens induce a less easily detectable CMI response than a similar antigen delivered in particulate form as shown by experiments in which either soluble or aggregated human serum albumin were used as the immunogen (Burrell and Hill, 1975; Hill and Burrell, 1979). Infectious organisms which replicate *in situ* are even more capable of producing CMI both locally and systemically (Waldman *et al.*, 1972; Spencer *et al.*, 1974; Ganguly and Waldman, 1972; Lipscomb *et al.*, 1982). Haptens, such as trinitrobenzene (Stein-Streilein, 1983), or metals (Parker and Turk, 1978), such as beryllium oxide (Haley *et al.*, 1989), may also induce CMI in the lung following direct instillation into appropriate animal models. Haptens and metals are agents that lead to sensitization of humans exposed to these agents in the workplace and are associated with hypersensitivity reactions.

A number of early studies addressed the issue of whether subcutaneous, iv, or direct lung instillation of antigens resulted in differences in the expression of CMI. In three separate studies, guinea pigs injected via the lung with human γ-globulin (HGG), DNP–HGG, or heat-killed influenza virus accumulated specific T cells among lymphocytes recovered from the lungs as measured by migration inhibition factor release or by antigen-induced lymphoproliferation assays. However, subcutaneous or iv injection of these antigens failed to result in measurable specific T cell accumulation in the lungs, although systemic CMI could be measured in lymphocytes from nodes draining the subcutaneous inoculation site or from spleen (Waldman and Henney, 1971; Nash and Holle, 1973; Lipscomb *et al.*, 1982). However, the initiation of a mild inflammatory response in the lungs resulted in the accumulation of immune T cells in the lungs of the animals immunized via the extrapulmonary route (Waldman *et al.*, 1972; Nash and Holle, 1973; Lipscomb *et al.*, 1982).

Using a live, attenuated rubella vaccine or another live strain of rubella virus, the kinetics of a local CMI response was studied following the inoculation of guinea pigs either subcutaneously or by an intranasal inoculation (Morag *et al.*, 1974). In these studies, migration inhibition factor activity was the parameter for measuring CMI responses and was initially detected in the lungs 2 weeks after immunization, peaked at 4 weeks, but was no longer detectable by 6 weeks.

When primary immune responses are generated in the LALN, what

stimulus recruits cells back to the lungs? Lymphoblasts rapidly exit lymph nodes during developing immune responses (Joel and Chanana, 1987). These cells enter the circulation and, under the control of adhesion molecules and locally generated chemotactic and other adhesion molecule-stimulating cytokines, are recruited into inflamed lungs (Berman *et al.*, 1990). As previously discussed, although markedly inflamed lungs nonspecifically recruit both T cells and B cells, memory T cells generated in LALNs may also have homing molecules that specifically direct their return to the lung (Picker *et al.*, 1994). An important factor in retaining recruited immune-specific cells is the continued presence of specific antigens within tissue and at least two groups have shown that this can occur (Lipscomb *et al.*, 1982,1983; Lyons and Lipscomb, 1983; Emeson *et al.*, 1982). Thus, T cell blasts enriched for two different antigens and labeled with two distinguishable radioisotopes were shown to be retained in lung lobes nonspecifically, but with an additional selectivity in lung lobes containing the relevant antigen (Lipscomb *et al.*, 1982). Selective retention was induced by antigen carried by APC deposited in the lungs and was class II MHC restricted, suggesting that the T cells were retained in the lung lobes by binding to the APC *in vivo* (Lyons and Lipscomb, 1983). These studies added validity to a concept that four general mechanisms relate to recruitment and retention of immune cells in the lungs: (1) recently activated T and B cells, i.e., lymphoblasts, from any lymphoid tissue are nonspecifically recruited into inflamed lungs; (2) cells recently activated in LALNs express adhesion molecules that are uniquely designed to target their binding to lung endothelium; (3) matrix and lung parenchymal cell adhesion molecules expressed under the control of local environmental perturbations facilitate the emigration of immune cells; and (4) antigen expressed on the appropriate MHC in the lung leads to preferential retention and further expansion in the lung. Specificity of recruitment for B cells has not been shown (see below), but in the presence of retained antigen and specific T helper cell recruitment, specific B cells could divide and differentiate.

C. B Cell-Dependent Lung Immunity

1. *Primary Immune Responses*

Antigen-specific antibody produced in LALNs after a primary lung immunization is released into blood (Bice *et al.*, 1980a; Shopp and Bice, 1987). In addition, large numbers of antigen-specific IgG, IgA, and IgM AFCs produced in LALNs also enter the blood after lung immunization of sevral species, e.g., dogs, cynomolgus monkeys, chim-

panzees, humans (Bice *et al.*, 1980a,1982b; Kaltreider *et al.*, 1981; Mason *et al.*, 1985; Weissman *et al.*, 1994). In studies of larger animals, AFCs in blood are recruited into a lung lobe exposed to antigen, but significantly fewer AFCs are found in the other lung lobes of the same animal that are exposed to saline or nothing.

There appear to be two factors that control the entry of AFCs into the lung. First, AFCs must have been recently produced in an immune response (Bice *et al.*, 1989). The lymphoid tissue in which they are produced exerts no control on their entry into the lung because AFCs produced in the popliteal lymph nodes enter the lung at the same rate as AFCs produced in LALNs (Hillam *et al.*, 1985). Second, AFCs enter sites of inflammation produced by instillation of antigen into the lung (Bice *et al.*, 1982a; Hillam *et al.*, 1985). However, the recruitment of AFCs into inflammatory sites in the lung is not antigen specific because they also enter lung lobes inflamed by instillation of particles or other inflammatory agents.

Plasma cells are found in the alveoli and interstitial lung tissues of immunized lung lobes suggestig that AFCs that enter the lung mature to plasma cells (Bice *et al.*, 1987a). Most antigen-specific IgM, IgG, and IgA antibody in the lung after a primary exposure to antigen is produced locally by these cells (Hill *et al.*, 1983). The few AFCs in control lung lobes exposed to saline also actively produce antigen-specific antibody (Bice *et al.*, 1980a,1989). The results of several studies show that AFCs in the lung after a primary immunization are recruited into the lung and are not produced locally (Mason *et al.*, 1985; Bice *et al.*, 1989). In addition, cell numbers are not amplified by interaction with antigen that might have been retained in the lung after primary immunization (Bice *et al.*, 1982a). In addition to AFCs, large numbers of other lymphocytes enter the lung with a peak response occurring between 7 to 14 days after immunization with a mean of 25% of the total lavage cells being lymphocytes (Bice *et al.*, 1989).

Species differences exist in the release of AFCs into the blood from LALNs and in the recruitment of AFCs into the lung. Dogs (Bice *et al.*, 1982b), nonhuman primates (Bice *et al.*, 1982a, Mason *et al.*, 1985), and humans (Stevens *et al.*, 1979; Lue *et al.*, 1988; Weissman *et al.*, 1994) all have large numbers of AFCs in their blood after immunization, and blood AFCs enter the lung. In contrast, data from a single immunization of the lungs of rats, guinea pigs, rabbits, and mice suggest that few or no AFCs are released into blood, and that relatively few AFCs appear subsequently in the lung (Bice and Shopp, 1988). However, the use of adjuvants and large doses of antigen appears to increase the number of AFCs in the lung of guinea pigs and mice

(Shopp and Bice, 1987; Curtis and Kaltreider, 1989). It is possible that the strain of species being evaluated may also be important, although no data are available that compare pulmonary B cell responses in different strains of laboratory animals.

2. *Memory Responses*

Although primary immune responses are not produced in the lung independently of secondary lymphoid tissues, data suggest that memory responses may be detected in the lung to antigen challenges that are independent of LALNs (Mason *et al.*, 1985; Jones and Ada, 1986, 1987; Bice *et al.*, 1991). The most logical explanation for the production of AFCs and antibody in the lung after an antigen challenge is that immune memory cells are recruited into and/or develop in the lung after a primary immunization. Unlike a primary immune response, most specific IgG and IgA antibody produced in an immunized lung after a rechallenge with antigen appears to come from local immune memory B cells and localized production of AFCs, rather than by AFCs recruited into the lung from blood. Only minimal specific IgM is produced in the lung after antigen rechallenge (Bice *et al.*, 1991).

3. *Long-Term Antibody Production in the Lung*

Specific antibody continues to be produced in the lung for several years after the last exposure to antigen (Bice *et al.*, 1991). Lavage fluid from immunized and challenged lung lobes contained significantly more specified IgG several years after the last exposure to antigen than was present in lavage fluid from control lung lobes. Thus, once an intense, localized antibody response was established in the lung, immune mechanisms supported continued localized antibody production for several years after the last exposure to antigen, but only at the site of antigen exposure.

Although AFCs were identified in lavage fluid from exposed lung lobes several years after antigen challenge, it was possible that cells in interstitial lung tissue, as well as in LALNs or distant lymphoid tissues, were all important in long-term antibody production. However, the evaluation of antibody production in lung and various extrapulmonary tissues showed that most long-term antibody production occurred in interstitial tissue in the immunized lung lobe (Bice *et al.*, 1993). Cells from control lung lobe tissue, from LALNs that received lymphatic drainage from the immunized lung lobes, from spleen, gut-associated lymph nodes, or popliteal lymph nodes did not produce significant levels of antibody 2 years after the last antigen challenge. Therefore, immune cells retained in lung tissue previously exposed

to antigens may be an important source of antibody to protect the lung. In addition, the absence of antibody production in LALNs or in other distant lymphoid tissue suggests that antibody produced in lung tissue exposed to antigen could possibly enter the bloodstream and provide immune protection for unexposed lung lobes and extrapulmonary tissues.

Two possible mechanisms could be responsible for long-term antibody production in lung lobes previously exposed to antigen. First, antigen retained in the lung, possibly on follicular dendritic cells, could stimulate antibody production by antigen-specific memory B lymphocytes that migrate through the lung. Alternatively, B lymphocytes recruited into or produced in the lung in response to the initial antigen challenge might live for several years and continuously secrete antibody. Because continuous antibody production occurs only in lung lobes exposed to antigen, an antigen depot may be essential. Data have been published that support both possibilities (Tew *et al.*, 1990; Peeters and Carter, 1981).

In summary, studies suggest that pulmonary humoral immunity can be maintained both by continued long-term spontaneous antibody production and by antigen challenge restimulating local pulmonary memory B cells to secrete antibody.

IV. Models for Immunity in Lung Infections

Despite the ability of the lung to express both natural and acquired immunity, respiratory tract infections are the most common type of infections experienced by humans. Certainly the common cold alone wins this competition hands down! Vaccination has been a powerful intervention to protect against many respiratory tract infections including the bacteria *Bordetella pertussis, Corynebacteria diphtheriae, Hemophilus influenzae, Streptococcus pneumoniae,* and *M. tuberculosis* and viruses, including one virus that primarily infects the respiratory tract, i.e., influenza, and several which initially infect via the respiratory tract, rubeola, rubella, and mumps. A major impetus for current research in infectious diseases is to learn more about natural host defenses in infections and how the immune system amplifies these defenses (Mason and Nelson, 1992). By better understanding these strategies, the hope is to optimize vaccination of immunocompetent hosts and perhaps even hosts who are immunosuppressed, yet retain some capacity to respond immunologically. Alternatively, in immunosuppressed individuals, once we are better able to understand how immunologically derived cytokines function in the normal host

during infections, recombinant forms might be administered as replacement.

The use of animal models has considerably enhanced our understanding of lung infections and the role of immunity in controlling them. In most pneumonias caused by extracellular bacteria, recruited phagocytes and opsonins, especially antibody and complement, are required to effectively control infections, even when antibiotics are used. Thus, the goal for vaccination in these pneumonias is to raise the level of local antibody. For chronic pneumonias and pneumonias caused by intracellular microorganisms, the goal of vaccination is less clear, although protection against the viruses listed previously correlates with serum antibody levels. The best evidence supports the probability that enhancing CMI would be protective for immunization against many obligate and facultative intracellular bacteria, fungi, and parasites (Lipscomb, 1989; Campbell, 1993).

In this section, examples of animal models of infectious disease that address how pulmonary immunity develops to various etiologic agents are discussed, and the type(s) of immunity that afford protection are indicated. While investigators have used experimental models to study nearly all of the infectious agents that produce respiratory infections, space dictates that only a few representative studies be included here.

A. Acute Bacterial Pneumonias

1. *Streptococcus pneumoniae*

The administration of *S. pneumoniae* into the lung leads to a rapid accumulation of neutrophils in the alveolar space. The development of this local inflammatory response during *S. pneumoniae* pulmonary infections was noted in early histopathological studies (Loosli, 1940; Wood, 1941; Loosli, 1942). Subsequent studies confirmed the requirement for intact granulocyte function in the host to eradicate the pulmonary infection (Wood *et al.*, 1946; Heidbrink *et al.*, 1980). Studies have examined the mechanisms responsible for neutrophil recruitment during the early stages of *S. pneumoniae* pulmonary infection (Vial *et al.*, 1984; Bruyn *et al.*, 1992). Several groups demonstrated that animals systemically decomplemented with cobra venom factor had an impaired ability to recruit neutrophils and to clear the organisms from the bronchoalveolar space. A role for C5 in the recruitment of neutrophils in response to intratracheally delivered *S. pneumoniae* was assessed by using congenic C5-sufficient ($C5^+$) and C5-deficient ($C5^-$) mice (Toews and Vial, 1984). The results indicated that C5 was important in producing optimal, early neutrophil recruitment and bacte-

rial clearance in response to *S. pneumoniae*, but other chemotaxins must be involved, because chemotactic activity and neutrophil recruitment was found in both $C5^+$ and $C5^-$ mice.

Once phagocytic effector cells were recruited into the lung, neutrophils and macrophages required the opsonins, immunoglobulin and complement, for efficient phagocytosis of the *S. pneumoniae* (Guckian *et al.*, 1980; Coonrood and Yoneda, 1981). The presence of C3b on the surface of the pneumococci is vital for phagocytosis. C3b can be deposited on *S. pneumoniae* by either the classical complement pathway through interaction with antibodies or through the alternative complement pathway (Winkelstein, 1981; Joiner *et al.*, 1980). *Streptococcus pneumoniae* that are not killed by the initial pulmonary inflammatory reaction drain to LALNs and eventually enter the systemic circulation resulting in a bacteremic phase (Austrian, 1981). During this phase, type-specific antibody to capsular polysaccharide is produced. It has been demonstrated through passive immunization studies that the presence of type-specific antibody (IgG and IgM) in the serum is protective against severe pneumococcal infection (Musher *et al.*, 1990). Nontype-specific antibodies to *S. pneumoniae* are made during infection, including antibodies to cell wall components (Brown *et al.*, 1983), surface protein A (Szu *et al.*, 1983), and the F polysaccharide (Au and Eisenstein, 1981). In general, most animal studies indicate that these latter antibodies play a minimal role in providing effective protection against infection by *S. pneumoniae* (Szu *et al.*, 1986; Brown *et al.*, 1983).

The role of secretory IgA in the prevention of pneumococcal disease is unclear, although one mouse model demonstrated that *S. pneumoniae*-specific IgA could "arm" lung lymphocytes which subsequently demonstrated antibacterial action against *S. pneumoniae* (Sestini *et al.*, 1988). IgA has been reported to fix complement and act as an opsonin (Hiemstra *et al.*, 1988; Gorter *et al.*, 1989) and thus could play a role preventing *S. pneumoniae* infection. Finally, humoral factors other than antibody have been implicated in protection against *S. pneumoniae*, particularly C-reactive protein which can activate complement and act as an opsonin when bound to the capsule. Although a role for C-reative protein has been demonstrated in clearing *S. pneumoniae* from the bloodstream (Volanakis and Kaplan, 1971; Horowitz, *et al.*, 1987), a role for C-reactive protein in the pulmonary stages of infections has not been demonstrated.

2. *Haemophilus influenza*

Models for acute pulmonary infection with *H. influenza* have been developed in the rat (Wallace *et al.*, 1989) and mouse (Esposito and

Pennington, 1984; Toews *et al.,* 1984b). The latter method delivers a reproducible bolus of organisms to the lower respiratory tract via an endobronchial catheter. Using this technique it was determined that the clearance of both typable and nontypable *H. influenza* from lungs occurred at a very similar rate. The clearance of the organisms appeared to occur in two phases. During the initial 6 hr postinoculation, the organisms increased in numbers three- to fivefold, while during the next 18 hr the organisms were rapidly cleared (Toews *et al.*, 1984b). Studies indicated that the rapid clearance phase corresponded to the influx of neutrophils into the lung and that the presence of these leukocytes was vital for effective clearance (Toews *et al.*, 1985). The effect of specific antibody to the *H. influenza* on the rate of pulmonary clearance was examined using both active immunization and passive administration of immune sera. The results indicated that the presence of specific antibody in the serum and the BAL fluid of immunized mice correlated with an increased rate of clearance from the lung. That systemic IgC could provide protection in the lower respiratory tract of animals was also demonstrated by experiments showing enhanced clearance of *H. influenza* from the lungs of mice that had received immune sera. Taken together, these results indicated that in the presence of elevated titers of serum IgG, protective antibodies could enter the airways of infected lungs to provide protection against pulmonary pathogens.

3. *Staphylococcus aureus*

In contrast to the organisms discussed previously, previous immunization with *S. aureus* does not appear to enhance clearance or provide protective antibody in pulmonary infections (Jakab, 1976). Recent studies suggest that the pulmonary clearance of this organism may be dependent on locally produced opsonins that enhance phagocytosis by AM. Surfactant protein A, produced by type II pneumocytes, can bind to *S. aureus* and increase phagocytosis, while this protein does not enhance uptake of *S. pneumoniae* by AM (McNeely and Coonrood, 1993).

B. Chronic Bacterial and Fungal Pneumonias

Lung infections with two microorganisms, Mtb and *Cryptococcus neoformans* (Cne), are discussed in this section as examples of infections requiring intact CMI for resolution. Animal models of chronic lung infections with several other important pathogenic organisms have been studied, including *Pneumocystis carinii* (Walzer, 1984; Shellito *et al.*, 1990; Harmsen and Stankiewicz, 1990; Boylan and Current, 1992), *Histoplasma capsulatum* (Baughman *et al.*, 1986; Defaveri and

Graybill, 1991; Fojtasek *et al.*, 1993; Allendoerfer *et al.*, 1993), *Blastomyces dermitiditis* (Morozumi *et al.*, 1982; Moser *et al.*, 1988; Frey *et al.*, 1989; Williams *et al.*, 1994), *Paracoccidiodes braziliensis* (Brummer *et al.*, 1984; Defaveri *et al.*, 1989), *Coccidiodes immitis* (Cox *et al.*, 1988), *Chlamydia trachomatis* and *psittaci* (Williams *et al.*, 1988), *Rhodococcus equi* (Kanaly *et al.*, 1993), and *Mycobacterium avium-intracellulare* (Takashima and Collins, 1988). Although infection with *Legionella pneumophila* can cause an acute pneumonia in susceptible hosts, it is a facultative intracellular bacterium; CMI is thought to be necessary for resolution of the infection. An animal model to study this infection has also been developed (Skerrett and Martin, 1991).

A central role for CMI (in which T cells recruit and activate macrophages) in controlling intracellular bacterial infections was first proposed by George Mackaness using a *Listeria monocytogenes* murine infection model (Mackaness, 1964). After finding an important role for CMI in controlling an aerogenous *Listeria* infection in mice, Mackaness extended his studies to propose that cell-mediated hypersensitivity might be an important cause of lung disease (Mackaness, 1971). However, he and his collaborators observed that, in contrast to protection afforded by active immunization, adoptive transfer of *Listeria* immune splenocytes seemed to afford only minor protection against an aerosolized infection (Truitt and Mackaness, 1971). In retrospect, adoptive immunity might have been transferred more successfully if LALNs or lung lymphocytes from aerosol-infected mice had been used instead of spleen cells from systemically immunized mice; these latter cells likely homed inefficiently to the infected lung (Huffnagle *et al.*, 1991b). Nevertheless, in experiments with virulent Mtb, when organisms were given iv, the lung developed effective resistance, although less effectively than spleens and livers (Mackaness, 1971). These early studies suggested that CMI in the lung might be more rigidly downregulated, perhaps to prevent excessive damage to delicate structures. However, Mackaness offered an additional explanation, e.g., lung infections with Mtb may be more difficult to control locally because organisms are sequestered in AM, macrophages that, in contrast to recruited monocytes, might resist activation signals delivered by T cells.

1. *Mycobacterium tuberculosis*

The role of pulmonary immunity during Mtb infection has been analyzed in a variety of animal models (Smith and Wiegehaus, 1989) including rabbits (Lurie, 1964), mice (Orme and Collins, 1984; North and Izzo, 1993), and guinea pigs (Smith and Harding, 1977). Initial

experiments involved intranasal or intratracheal inoculation and the development of the Middlebrook chamber (Middlebrook, 1952) provided a means of aerosolizing Mtb into animals. Early studies examined the number of Mtb required for a reproducible infection in animals. Mice exposed to a mist of virulent Mtb developed discreet lesions that were progressively fatal over a 10–21 week period (Schwabacher and Wilson, 1937). These initial studies were extended by comparing aerosol versus intranasal delivery of Mtb and it was found that both routes produced similar pathology. It was observed that a deposited inoculum of about 100 organisms was required for reproducible infection, while a dose of approximately 12,000 organisms resulted in death (Glover, 1944).

Resident AM undoubtedly play a role during an Mtb infection. Mtb deposited into the lung are rapidly taken up by AM. Evidence for a role for AM in defense against Mtb partly comes from epidemiologic studies examining Mtb infections in individuals with silicosis (Snyder, 1978). Silica exposure results in the uptake of silica particles by AM. These silica particles remain in the phagolysosomes of AM throughout the life of the individual (Allison and D'Accy Hart, 1968) and likely affect their function. Essentially all epidemiological studies examining the incidence of Mtb infections in a silica-exposed population have concluded that the incidence of Mtb infections in this group is significantly higher than the incidence of Mtb infection in a non-silica-exposed population (Snyder, 1978).

Several studies have shown that both human and/or mouse AM are stimulated to produce chemotactic factors and cytokines in response to Mtb or components of the Mtb cell wall (Barnes *et al.*, 1992; Chatterjee *et al.*, 1992). These released products may represent an early native defense system against Mtb. Thus, chemotactic factors can act to recruit neutrophils and monocytes from the circulation, while AM-released cytokines, such as TNFα, can activate both local AM and newly recruited cells. Indeed, some studies indicate that cytokine-activated AM and/or monocytes can inhibit the growth of or kill Mtb (Crowle, 1990; Rastogi, 1990; Denis, 1991a). More recent data suggest that macrophage cytokines, including IL12, may enhance the development of the Th1 subset leading to protective immunity (Hsieh *et al.*, 1993). Data indicating that avirulent Mtb can elicit a greater cytokine response from macrophages than virulent Mtb have lead to the hypothesis that the observed differences in Mtb virulence may be due to an intrinsic ability of virulent Mtb to prevent or decrease the release of factors by AM (Barnes *et al.*, 1992; Chatterjee *et al.*, 1992; Roach *et al.*, 1993). Ethnic differences observed in susceptibility to Mtb (Coultas *et*

al., 1993) might be due to a genetic disposition for a poor initial response by AM to Mtb.

A role for CMI was demonstrated for protection against Mtb infection (Suter, 1961; Leveton *et al.*, 1989). In a guinea pig model, it was demonstrated that in animals given a low dose of Mtb, the organisms replicated in a log phase until Days 19 or 20, after which exponential growth ceased (Smith and Harding, 1977). The decrease in growth coincided with the onset of tuberculin skin test sensitivity and the development of detectable bacillemia. Bacteriostasis ensued over the next 40–50 days after which the numbers of Mtb in lung were gradually reduced. Although these data were consistent with a role for the development of an acquired CMI response for resolution of the Mtb infection, it was not until Orme and Collins (1984), by examining the immune response in a mouse model, that direct evidence was provided for a role of T cells in pulmonary immunity against Mtb. In a series of adoptive transfer experiments, they removed splenic T cells from a mouse that had received an iv inoculation of *Mycobacterium bovis* or BCG. After injecting these BCG-immune T cells into thymectomized, sublethally irradiated nonimmune mice, the mice were challenged with an aerosol dose of Mtb that deposited 10^4 organisms into the lungs. Two important findings in these studies were (1) adoptively transferred immune T cells enhanced clearance of Mtb from the lung; and (2) by differentially removing subsets of T cells with specific antisera, the skin test tuberculin sensitivity was dissociated from protective antituberculous immunity which indicated that separate populations of T cells may be responsible for the two events. In a follow-up study (Orme, 1987), only Mtb-immune CD8 T cells adoptively transferred protection to mice challenged with a lethal aerosol inoculum (1.5×10^5 organisms) of Mtb, while either CD4 or CD8 cells could transfer protection to mice exposed to a low dose (500 organisms) of Mtb.

In comparison to the pulmonary inoculation studies, models using intraperitoneal or iv routes of inoculation have produced different results. In an *intraperitoneal* model, an Mtb-reactive CD4 T cell clone provided both a DTH response and protection as measured by the growth of Mtb in the peritoneum (Pedrazzini and Louis, 1986). Similarly, an Mtb-immune CD4 T cell clone provided protection, as measured by reduced splenic CFU, following an iv Mtb infection. Another study using *in vivo* depletion of T cell subsets demonstrated that depletion of CD4 T cells decreased resistance to iv infection, while depletion of CD8 T cells did not have a significant effect (Pedrazzini *et al.*, 1987). In contrast, in a similar model, transgenic mice incapable

of producing CD8 T cells were shown to have a decreased resistance to Mtb compared to normal mice (Flynn *et al.*, 1992).

A role for $\gamma\delta$ T cells in pulmonary defenses against Mtb is unresolved (O'Brien *et al.*, 1989; Kaufmann and Kabelitz, 1991). Because $\gamma\delta$ T cells release IFNγ, it is tempting to speculate that these T cells represent an initial defense mechanism in the lung to provide activating cytokines to enhance local effector mechanisms to help control the infection until the development of protective immunity by $\alpha\beta$ T cells. Initial studies demonstrated an increase in the number of lung $\gamma\delta$ T cells after an intratracheal dose of PPD (Janis *et al.*, 1989). Other studies suggested that many $\gamma\delta$ T cells responded to the heat-shock protein of Mtb (Born *et al.*, 1991; Kaufmann and Kabelitz, 1991). Other studies suggested they may play a role in granuloma formation (Modlin *et al.*, 1989). However, in humans with active Mtb infections, there was no increase in $\gamma\delta$ T cells in the granuloma as determined by immunohistochemical staining (Tazi *et al.*, 1991). More work in animal models and human natural infections is required to define the role of $\gamma\delta$ T cells in mucosal immunity, particularly regarding their role in Mtb infections.

Complex interactions exist in the development of protective immunity by T cells and the type of cytokines produced during an infection. Similar to the studies that show an important protective role for Th1 cells that preferentially secrete IFNγ in *Leishmania* infections (Locksley *et al.*, 1991), it is likely that mechanisms for production of appropriate cytokines are critical in the development of protective immunity against Mtb (Flesch, 1990; Denis, 1991b; Kawamura *et al.*, 1992; Barnes *et al.*, 1993; Orme *et al.*, 1993) as well as in the maintenance of a resistant state during the chronic infection stage. An important role for IFNγ in Mtb resistance was recently demonstrated in both an aerosol and an iv Mtb infection model. Comparing infected normal and IFNγ knockout mice (Cooper *et al.*, 1993; Flynn *et al.*, 1993), it was demonstrated that a lack of IFNγ resulted in a significant increase in Mtb susceptibility. However, since IFNγ was absent throughout the course of infection, it was unclear at what stage in the immune response IFNγ was required (Flynn *et al.*, 1993). Indeed, IFNγ may be important for all aspects of the response to Mtb including T cell development, cell recruitment, and activation of effector mechanisms.

Other studies in mice examined the granulomatous response to iv-injected BCG in animals that had received neutralizing antibody to TNFα (Kindler *et al.*, 1989). These and other studies (Amiri *et al.*, 1992) indicate that TNFα also plays a critical role in protection against Mtb, particularly in the development and maintenance of granulomas.

Attempts to vaccinate animals with avirulent or killed Mtb have

provided important data regarding potential vaccines. In general, studies suggest that to enhance the immune response against virulent Mtb, viable organisms must be used (Larson and Wicht, 1962). The route of immunization with viable organisms can be either iv or by aerosol. Further, vaccination does not prevent infection, but rather limits tissue destruction and the degree of hematogenous dissemination (Harding and Smith, 1977). Immunization with nonviable cellular elements does not afford protection.

In summary, the development of protective immunity to a pulmonary infection with Mtb requires the coordinated activity of multiple cell types, particularly macrophages and T cells. The continued study of the Mtb pulmonary infection should aid in understanding the mechanisms for developing effective CMI in the lung and suggest strategies to enhance pulmonary defenses.

2. *Cryptococcus neoformans*

Cne is an encapsulated yeast found in desiccated form in soil, particularly in areas contaminated by pigeon feces. Cne usually causes only an asymptomatic infection in humans following inhalation. Normal individuals typically clear the organisms, but in those who are susceptible, particularly those with defects in CMI, the organism may disseminate via the bloodstream and produce an extrapulmonary infection, usually meningitis. Mice have been used as experimental models for studying the host defenses against this microorganism, which because of the capsule resists endocytosis and thus typically replicates in tissues in an extracellular location. In murine models the organism was frequently inoculated iv or ip, although it had been established many years ago that mice housed on contaminated bedding (Smith *et al.*, 1964), exposed to aerosols (Karaoui *et al.*, 1977), or that received intranasal inoculations of the organism (Ritter and Larsh, 1963) developed infection. These early studies validated the concept that the organism was acquired by the respiratory tract. Murphy and her colleagues have contributed substantially to the understanding of immune and natural defense mechanisms in protection against this yeast and demonstrated that animals inoculated intranasally developed pulmonary infection that disseminated, but following the development of DTH gradually cleared the infection (Lim *et al.*, 1980a). Furthermore, these investigators demonstrated that transfer of T cell-enriched splenocytes from mice immunized by an intranasal infection was capable of protecting mice against an iv challenge (Lim *et al.*, 1980b). Of importance was that in these studies, passive transfer of serum failed to protect mice.

We and others developed an intratracheal inoculation infection

model with Cne to study pulmonary immune mechanisms in mice (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991a; Huffnagle and Lipscomb, 1992). In our model, the organism is inoculated in small amounts directly into the trachea and yeasts in the lungs are quantitated by homogenizing the organ and measuring colony-forming units (CFU). Over an initial 7 days, the organisms grow rapidly followed by a gradual decrease in CFU in appropriate mouse strains, a process referred to as "lung clearance" (Huffnagle and Lipscomb, 1992). An important aspect of this model, as is true of many other lung infection models, is that both the strain of microorganism and the strain of mouse determine whether the infection will be cleared from the lung and at what rate (Huffnagle *et al.*, 1991a). In studies using a relatively low virulence encapsulated yeast, athymic nude mice, mice with severe combined immunodeficiency (SCID), or mice depleted of CD4 and CD8 T cells were incapable of pulmonary clearance (Huffnagle *et al.*, 1991a,b; Huffnagle and Lipscomb, 1992; Hill and Harmsen, 1991). Furthermore, protection of the lung was adoptively transferred to SCID mice by splenic lymphocytes, but adoptive immunity was more effective if lymphocytes isolated from the lungs and LALNs of animals that had been immunized during a lung infection were used (Huffnagle *et al.*, 1991b). Interestingly, using a more virulent organism, CD4 T cells were responsible for increased resistance to the highly virulent organism following extrapulmonary spread, but did not demonstrate an effect in controlling the infection within the lung (Mody *et al.*, 1990). These latter studies demonstrated, as did the earlier studies of Mackaness, a dichotomy between the ability of immunized animals to demonstrate effective immunity in the lung compared to extrapulmonary organs.

What is the role of CD4 and CD8 T cells in immune protection? The absence of either reduced the numbers of inflammatory cells, including macrophages, but they were even more profoundly decreased when both were absent (Huffnagle *et al.*, 1994). A role for CD8 T cells was repeatedly shown in strains of mice that demonstrated acquired resistance to low-virulence Cne, i.e., BALB/c, C.B-17 (congenic to BALB/c, but with the IgH locus of C57BL/6 mice), and CBA mice (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991a; Mody *et al.*, 1993). A role for CD8 T cells in this infection was particularly curious, although CD8 cells clearly participate in the development of immunity to intracellular organisms. Mechanisms proposed include secretion of IFNγ or lysis of infected targets following recognition by CD8 T cells of peptides in the context of class I MHC (Kaufmann, 1988). However, it is not known how CD8 cells function in murine Cne disease in which

the organism is primarily extracellular. One important mechanism may be related to their capacity to enhance either the clonal expansion or the recruitment of CD4 cells to the lung (Huffnagle *et al.*, 1994). Thus, CD8 T cell depletion of Cne-infected mice reduced the numbers of CD4 T cells in infected lungs. A second role may relate to the finding that lung cells isolated from CD4 T cell-depleted animals were capable of secreting IFNγ in mitogen-stimulated cultures, suggesting CD8 cells in this setting could also contribute to IFNγ production and play a role in macrophage activation. It was also demonstrated that CD8 cells played a critical role in the development of DTH to Cne in Cne-infected mice and could adoptively transfer DTH (Mody *et al.*, 1994). This is an important observation because it proves CD8 T cells can recognize antigens of extracellular organisms in the context of class I MHC. Thus, it is possible that CD8 T cells might lyse AM that phagocytose the organism, but cannot kill it, so that activated macrophages or other effector cells may play a role.

C.B-17 mice were particularly adept in the development of a Th1 response characterized by clearing Cne from their lungs, and this ability was related to enhanced secretion of IL-2 and IFNγ by LALN cells early in infection (Hoag *et al.*, 1994). The heightened resistance in C.B-17 mice correlated with expression of the inducible nitric oxide synthase (iNOS) gene in the lungs, was accompanied by secretion of NO by lung cells during the early clearance phase, and was completely abrogated by both anti-IFNγ treatment and feeding animals an inhibitor of NO production (Lovchik *et al.*, 1995). Thus, in C.B-17 mice, clearance in the lung was related to the capacity of the animals to make IFNγ and NO.

If T cells are necessary to protect lungs from *Cne* infections, is the effector mechanism mediated mainly by activation of macrophages? The answer to this question is still uncertain, but rat AM activated by IFNγ were able to inhibit the growth of Cne (Mody *et al.*, 1991). Furthermore, prolonged incubation with GM-CSF also activated AM for Cne growth inhibition (Chen *et al.*, 1994). Murine macrophages from the peritoneum of BCG-immunized mice inhibited the growth of Cne *in vitro* by an arginine-dependent mechanism and were related to NO production (Granger *et al.*, 1988; Alspaugh and Granger, 1991) consistent with the *in vivo* data of Lovchik (1995). An important aspect of growth inhibition by this NO-dependent mechanism was that it did not require endocytosis, although endocytosis enhanced the growth inhibition (Granger *et al.*, 1986). Others have demonstrated that IFNγ-activated mouse macrophages kill Cne, but determined that a secreted protein was important (Flesch *et al.*, 1989). Activated human macro-

phages make little if any NO unlike rat and mouse macrophages leaving open the question of what effector mechanism human macrophage may use to growth inhibit Cne.

Human neutrophils and macrophages not only inhibited growth, but killed, Cne in cultures that include fresh complement (Miller and Mitchell, 1991). The organism fixes complement by the alternate pathway resulting in C3bi binding to the yeast capsule and allowing phagocytosis by CR3-positive neutrophils and macrophages (Kozel and Pfrommer 1986; Kozel *et al.*, 1988). This mechanism was shown to play an important role in clearing Cne from the pulmonary vasculature during fungemic states in mice (Lovchik and Lipscomb, 1993). However, Cne in tissues tend not to provoke brisk inflammation, and bronchoalveolar spaces (and cerebrospinal fluid) do not contain significant complement. Furthermore, AM may not express CR3. Thus, T cells must amplify effector systems by recruiting and/or activating nonspecific effectors or by themselves becoming direct effectors.

Recent studies indicated that human NK cells and T cells had direct activity *in vitro* against Cne (Levitz *et al.*, 1994; Murphy *et al.*, 1993), although there was conflicting evidence that human NK cells had no growth-inhibiting activity unless antibody against the organism was present (Miller *et al.*, 1990). We demonstrated that murine NK cells had a minor effect against the organism following iv inoculation, but failed to play a role in *early* lung clearance if the organism was inoculated via the trachea (Lipscomb *et al.*, 1987). Recently a T cell-independent, partially protective host defense mechanism was found in lung clearance in SCID mice and BALB/c mice depleted of CD4 and CD8 T cells. A $Thyl^+$, $CD4^-$, $CD8^-$, asialo $GM1^-$ cell was responsible (Hill and Dunn, 1993). Further studies are clearly indicated to examine the role of this cell in animal models and to identify its origin, particularly in view of the possible importance of these cells in human Cne infections.

Histologic examination of murine lungs during the clearance phase demonstrated that yeasts were surrounded by macrophages with an activated appearance (Hill, 1992; Huffnagle and Lipscomb, 1992). This appearance was similar to that seen in the lungs of humans with cryptococcomas who are known to be able to resolve their infections without antibiotic therapy. When the lung becomes inflamed and complement is available, neutrophils may play some role in killing Cne in the lung, although the relative importance of neutrophils over T cells and activated macrophages remains to be clarified.

Continued studies using a murine Cne lung infection model should help elucidate the mechanisms that lead to the development of a

Thl-like response in LALNs early during infection and subsequent recruitment of lymphocytes and macrophages into the lung. A closer examination of what effector mechanisms are at work in the lungs of animals that clear a Cne infection, particularly in resistant mouse strains that may not utilize NO from activated macrophages, may help elucidate host defense mechanisms in man.

C. Viral Pneumonias

Viruses are intracellular organisms that usurp host cellular machinery to replicate. Viral entry into cells can be blocked by antibodies. However, once inside the cell, the virus is resistant to both antibody and T cell recognition until viral peptides are presented in the context of class I MHC antigens on the cell surface and allow specific cytotoxic T cells to lyse the infected cell (Zinkernagel, 1993). As viral replication ensues and particles are released from the cell surface, antibody again has an opportunity to block the further spread of the virus. In general, cytotoxic T cells play an important role in controlling local viral replication, while antibody can *prevent* initial infection and *extracellular* spread within the host. In some viral infections, there seems to be a relatively minor role for CD4 T cells (Zinkernagel, 1993). However, in a number of viral infections in mice, depletion of CD4 T cells increased mortality and reduced the rate of clearance. The role for CD4 cells may relate to providing help for cytolytic T lymphocyte (CTL) development (Reiss and Burakoff, 1981) and for B cell production of high-affinity IgG and IgA antibodies. The role of $\gamma\delta$ T cells, NK cells, and macrophages in acute viral infections is still not clear. Furthermore, whether memory CTLs play an important role in preventing recurrent infection is also uncertain (Zinkernagel, 1993). It is also uncertain whether persistence of long-term memory T or B cells against viruses requires the continued presence of virus or viral particles (Zinkernagel, 1993; Sprent, 1994).

A large number of viruses infect the respiratory tract, including rhinoviruses, coronaviruses, adenoviruses, influenza, and parainfluenza viruses, respiratory syncytial virus (RSV), measles, mumps, and rubella viruses. Good models in mice exist for both RSV and influenza A infections and are discussed to highlight experimental models that have provided insight into immune defenses against viral respiratory tract infections.

1. *Respiratory Syncytial Virus*

Immunization against influenza A with killed or fractionated viral antigens protects against influenza, but immunization against RSV has

been problematic (Salk and Salk, 1977; Wright *et al.*, 1982; Graham *et al.*, 1993; Alwan *et al.*, 1994). Protection against RSV, which produces a bronchiolitis in infants and is the most common cause for hospitalizing infants in Western countries, was not afforded by immunization with formalin-inactivated virus. Subsequent infection after such immunization sometimes resulted in unusually severe infections and even death (Kapikian *et al.*, 1969; Kim *et al.*, 1969). The mechanism is unknown, but various theories include immune complex disease, a CD4 T cell-mediated DTH reaction, or a CTL-mediated pneumonitis (Graham *et al.*, 1993). Recent efforts using RSV infections in mice have sought to understand what the mechanisms for protection might be, and why immunization might lead to enhanced pathogenicity with a subsequent challenge.

Depleting mice of either CD4 or CD8 cells reduced the disease in the lung following an initial RSV infection, but also enhanced virus replication (Graham *et al.*, 1991). Thus, control of viral replication during even a primary infection resulted in lung pathology. At least two groups attempted to determine whether various viral subunits might initiate protective immunity, yet cause minimal pathology. Mice vaccinated either parenterally or by intranasal inoculation, followed by nasal RSV challenge, lead to the expression of cytokine mRNA in the lungs (Graham *et al.*, 1993). The specific cytokine mRNA detected was dependent on whether live, heat-killed, or subunit vaccines were given. Inactivated virus or subunit fusion (F) protein induced cytokine expression that suggested a Th2-like lymphocyte response with increased IL-4 mRNA relative to IFNγ expression. In contrast, when mice were primed with parenteral or nasal live virus, Th1 responses were prominent. Formalin-fixed virus and the F protein component were somewhat protective. However, the most effective protection was induced by immunizing intranasally with the live virus. Furthermore, this immunization protocol resulted in the least lung pathology after rechallenge.

Experiments were designed to determine which T cell types caused pathology and whether specific RSV subunits evoked specific pathology-producing immune T cells. Cell lines were developed from immune lymphocytes of mice immunized against the F protein, the major surface glycoprotein (G), and a 22-kDa matrix protein expressed by recombinant vaccinia virus (Alwan *et al.*, 1994). F protein lead to the development of both CTL and CD4 T cells with a Th1 phenotype. G protein facilitated the development of CD4 cells with a Th2 phenotype. Immune cells from the 22-kDa protein-immunized mice resulted in predominantly CD8 CTL. Representative cell lines from each of

these groups transferred both protection and pathogenic effects to RSV-infected mice, but the Th2 cells seemed to be the most damaging. Furthermore, combinations of lines afforded the greatest protection. Thus, protection is often synonymous with pathology and it may be difficult to dissociate the two.

2. *Influenza Virus*

It has been clear for some time that serum antibody correlates with protection against influenza viruses. Influenza viruses exhibit antigenic drift and shift that requires individuals be immunized yearly for protection against the prevalent virus strain (Zinkernagel, 1993; Salk and Salk, 1977). While immunization against influenza has been successful, it is possible that new immunization protocols might be developed that would be broadly protective. In contrast to B cell epitopes, T cell epitopes may be cross-reactive; cytotoxic T cells seem to play an important role in controlling influenza infections (Zinkernagel and Althage, 1977).

Early studies established that recovery of mice from infections with influenza A required the development of a CTL response to the virus, and protection was afforded by the adoptive transfer of immune cells into naive-infected hosts (Yap *et al.*, 1978; Lukacher *et al.*, 1984). Further studies indicated that both class I and II MHC-restricted T cell clones could promote recovery from a lethal pulmonary infection (McDermott *et al.*, 1987). These T cell clones were preferentially retained in lungs of influenza-infected mice, independent of any viral antigenic specificity, and migrated from the pulmonary vessels into the bronchiolar lumens. Thus, the immune cells accumulated at a site appropriate to provide protection against a viral challenge.

Mice die within 6 days of lethal influenza infections. An array of cytokines could be detected in BAL fluids in these mice, but none that were unequivocally indicative of a T cell response (Hennet *et al.*, 1992). Thus, while IL-1α, IL-1β, IL-6, TNFβ, GM-CSF, IFNγ, and leukotriene B_4 were identified in lavages, IL-2, IL-3, and IL-4 were not. On the other hand, in sublethal infections in which the influenza infection was resolved, examination of cells from lavages as well as from LALNs demonstrated T cell cytokine production (Carding *et al.*, 1993; Sarawar *et al.*, 1993). In a primary infection, the kinetics of cytokine mRNA was compatible with an initial response occurring in the regional lymph nodes with the effector T cells appearing later in the lungs. Among the $\alpha\beta$ T cells, transcripts for IFNγ and TNFβ were predominantly found in CD8 cells, but there was a tendency for IL-4 and IL-10 to appear in CD4 cells. Interestingly, $\gamma\delta$ T cells were identified and expressed IL-2, IL-4, and IFNγ. During a secondary

response, T cell cytokine mRNA was found almost simultaneously in LALNs and in the lung (Carding *et al.*, 1993). In related studies, mRNA was detected by *in situ* hybridization and by cytokine production identified in individual cells by ELISPOT. The majority of cells in lavages produced IL-2, IL-4, and IFNγ with relatively little TNF and IL-10. Depletion of CD4 and CD8 cells caused a significant reduction in IL-2- and IL-4-producing cells, but IFNγ-producing cells remained and were likely $CD4^-$, $CD8^-$ αβ, or γδ T cells; both populations were present during the infection.

These studies are typical of recent studies examining cytokine patterns in various lung infections in the lung attempting to learn what determines the type of immune response that develops to airway antigens. B2-Microglobulin-deficient mice were used to determine the effect of IFNγ on influenza clearance. Mice did not develop CD8 CTL because of the absense of class I MHC, but residual CD4 cells were capable of mediating clearance, possibly related to the development of antibody of the IgG2a subclass (Sarawar *et al.*, 1994). Treatment of the mice with anti-IFNγ antibody delayed clearance for at least 3 days, whereas antibody to IL-4 had no effect. However, all mice survived and eventually cleared the virus. Notably, neither antibody to IFNγ nor IL-4 altered the cytokine profiles detected in freshly isolated lung lymphocytes. The conclusion was that although IFNγ played an important role in viral clearance, its role was not to drive CD4 cells to become Th1 T cells.

γδ T cells developed as a prominent component of the late inflammatory process during murine influenza infections (Carding *et al.*, 1990). These cells expressed all known γ genes, although some predominated at times. A suggested role for these cells was that they recognized heat shock proteins on inflammatory macrophages and decreased their numbers. However, close examination of the data failed to show an inverse relationship of the numbers of γδ T cells and lung macrophages (Carding *et al.*, 1990). In another study, γδ T cells were found to be noncytolytic, but expressed mRNA for IFNγ, GM-CSF, and TNFβ (Eichelberger *et al.*, 1991). The hypothesis was presented that these cells, through their capacity to make cytokines, provided nonspecific protection against secondary infections. Thus, although γδ T cells are a component of the host response to viral lung infections, their role remains unknown.

D. Implications for Vaccination

A National Institutes of Allergy and Infectious Diseases (NIAID) Blue Ribbon Panel on Vaccine Research was convened in 1993 by Anthony Fauci, Director of the NIAID, to assess the long-term goals

for vaccine research and to recommend immediate priorities for the institute. Among the panel's recommended priorities was the development of vaccines for respiratory infections of children, and to improve the current vaccines for pertussis and measles. An additional priority was to develop vaccines for reemerging infectious diseases, including influenza because of its inherent problems of antigenic drift and shift.

The Jordan Report, a publication of the Division of Microbiology and Infectious Diseases of the NIAID, has reviewed on a yearly basis the progress in vaccine research. In the 1993 report, respiratory tract infectious diseases for which vaccines were either being developed or improved were listed and discussed. They included the bacteria Groups A and B Streptococci, *H. influenzae* type B, nontypable *H. influenzae, Neisseria meningitidis, S. pneumoniae, B. pertussis, Pseudomonas aeruginosa,* and *M. tuberculosis;* the viruses Rubeola, rubella, adenoviruses, influenza, and parainfluenza viruses, and respiratory syncytial virus; *Mycoplasma pneumoniae;* and the fungi, *H. capsulatum, C. immitis,* and *C. neoformans.* Thus, an enormous scientific effort is being directed at designing vaccines to protect the host from respiratory pathogens. And yet the report also highlighted the gaps in our knowledge about normal host defenses at mucosal surfaces, what immune defenses we should attempt to enhance with vaccinations, and the best methods for immunization.

Important issues for vaccine research have been discussed in recent publications (Lambert, 1993) including reviews on the possibilities of immunization against tuberculosis (Kaufmann and Young, 1992) and on novel approaches to vaccination such as inoculating polynucleotides encoding antigens directly into muscle (Donnelly *et al.*, 1994).

Important general goals for any vaccine are that it be efficacious, easy to store, easy to give, and be free of side effects. Specific goals for vaccines for respiratory infections are (1) the immune response generated must be protective (a corollary is that the immune response to a microbial challenge should not cause lung pathology.); (2) the immune response must be quickly available in the respiratory tract and at the site within the respiratory tract where the microorganism is likely to seek entrance and/or produce disease; and (3) immune memory should be long term.

The study of the type of protective immune responses that develop during natural infections in man or induced infections in experimental animals has provided important clues to what responses should be enhanced by immunization. This has been a productive approach for viral infections and those caused by intracellular pathogens. However, host responses that can prevent a second infection may differ from the

immune responses that bring an acute infection under control. For example, in many viral infections, although cytotoxic T cells may bring a primary infection under control, antibody may prevent reinfection. Another example of this principle is that although CMI controls *Cne* infections in mice, in special circumstances, antibodies to *Cne* can be protective (Mukherjee *et al.*, 1992). The isotype of the predominating antibody in preventing infection may also be extremely important, e.g., upper tract infections may be more dependent on IgA responses, while in the alveolar spaces, IgG may be more effective.

As already discussed, an important consideration for viral vaccines is whether subunit vaccines are as effective as live attenuated organisms in initiating the desired type of immune response. An innovative strategy for generating CMI responses against nonviable antigens is to inoculate a regulatory cytokine at the time of antigen delivery (Afonso *et al.*, 1994). The vaccine could even consist of a fusion protein of the antigen and the cytokine (Tao and Levy, 1993).

The immune response at the time of challenge must develop in the correct site. If the organism infects the upper respiratory tract, local IgA is important. If infection is initiated in the lower respiratory tract, it may be sufficient that protective cells and antibody are available in circulation if recruitment can occur immediately following challenge. However, if significant inflammation is required before recruitment of immune cells occurs, some clinical manifestations of infection must necessarily develop, before the protective response neutralizes the infection. Feasible strategies for generating IgA in the upper respiratory tract are to aerosolize the antigen, to deliver the relevant antigens on the surface of a nonpathogenic microorganism, such as *Streptococcus gordonii,* that have the potential to colonize the nasopharynx (Pozzi *et al.*, 1992), or to immunize with oral vaccines with antigens either chemically bound to cholera toxin B-subunit (McGhee *et al.*, 1992), enclosed in a liposome or biodegradable microsphere (Mestecky and Eldrigde, 1991), or encoded in a plasmid carried by live attenuated *Salmonella* spp. (Cárdenas and Clements, 1992). Ample evidence has documented that IgA precursors generated in the gut home to bronchial mucosa (Weisz-Carrington *et al.*, 1987; Chen *et al.*, 1987; Ruedl *et al.*, 1994), although the converse does not appear to occur to an appreciable extent (McDermott and Bienenstock, 1979; Joel and Chanana, 1987; VanCott *et al.*, 1994). However, a cautionary note in relationship to immunizations that enhance IgA responses is that they could also stimulate IgE responses and lead to allergic responses in the lungs. Oral immunization with protein antigen and cholera toxin resulted in anaphylaxis in mice following an intraperitoneal antigen challenge (Snider *et al.*, 1994).

An important issue for any vaccine is whether long-term memory is possible. Based on the data derived from immunization with subunit vaccines or killed microorganisms, it seems unlikely that sufficient T cell memory could be induced for long-term CMI protection against respiratory tract infections. However, in the presence of continuous antigen, such as would occur with low levels of replicating attenuated viruses or retained intracellular microorganisms in Ia-positive APC, memory T cells should persist. Furthermore, follicular dendritic cells are present in BALT, and long-term B cell memory could also persist due to the retention of nonviable antigens in the form of antigen–antibody complexes on these cells. Evidence that long-term B cell memory occurs in the lungs of dogs has been obtained as discussed previously. However, it is important to remember that this observation was made in animals in which the antigen was directly instilled into the lung. Therefore, it is not obvious that extrapulmonary immunization would induce retention of long-term local B cell memory, and suggests that lung immunity might be more effective if primary immune responses to pulmonary pathogens were boosted by intranasal or aerosol antigen delivery.

V. Models for Hypersensitivity Lung Disease

Animal models of human lung disease have been used to test hypotheses under well controlled conditions and to dissect mechanisms of injury, inflammation, and repair. These models have been particularly useful in distinguishing direct lung toxicity from injuries that result from immune mechanisms.

To prove that a lung injury is immune mediated requires previous exposure to an appropriate agent, evidence of a specific immune response, and evidence that the injury involves recognized immunologic mechanisms. Early work concentrated on dissecting isolated aspects of the immune response, i.e., the role of antibody, antibody plus complement, or T cells, emphasizing *in vivo* analogs of *in vitro* events. Current models of immune-mediated injury emphasize the interrelationships among these aspects of the immune response. In particular, the importance of T cells (especially CD4 T cells) in regulating the type of the immune response is better appreciated.

A. Early Models

Early studies of immune-mediated lung disease in animals were performed by administering different antigens via different routes and with different adjuvants to guinea pigs (Richerson, 1972). Immune

responses varied depending on the type of antigen, method of immunization, and the presumed predominant response. Animals immunized with ovalbumin (OVA) in complete Freund's adjuvant (CFA) developed specific complement-activating antibody and hemorrhagic pneumonitis with a predominant neutrophil response after aerosol antigen challenge. In contrast, animals immunized with ABA-*N*-acetyltyrosine in CFA exhibited DTH without demonstrable serum antibody. Later, OVA aerosol challenge in sensitized animals produced scattered focal areas of alveolitis with thickening and increased cellularity of alveolar septa and alveolar filling with mononuclear cells. Other work (Brentjens *et al.*, 1974) confirmed the hemorrhagic nature of immune complex-mediated lung disease. Hemorrhagic neutrophilic pneumonitis could be transferred with serum and suppressed by administration of cobra venom factor, which depletes C′ *in vivo* (Roska *et al.*, 1977).

Richerson and colleagues described the results of aerosol OVA exposure of rabbits systemically sensitized to OVA in CFA. Acute exposure lead to transient foci of acute pulmonary inflammation (Richerson *et al.*, 1971), whereas chronic exposure caused decreasing inflammation (Richerson *et al.*, 1978). T cells were prominent in both the acute and the chronic lesions (Upadrashta *et al.*, 1988). Inhalation of muramyl dipeptide could substitute for systemic immunization with OVA in Freund's adjuvant (Richerson *et al.*, 1982). The pulmonary inflammatory response could be decreased by administration of cyclosporin A at the time of aerosol challenge (Kopp *et al.*, 1985), implicating DTH in the etiology of pulmonary inflammation in the rabbit OVA model.

Diminution in the pathologic response in the lungs despite continuing challenge could be produced by either repeated iv or aerosol exposure of rabbits to OVA and was not associated with decreased antigen-specific lymphocyte proliferation nor decreased blood or BAL antibody response (Richerson *et al.*, 1981; Butler *et al.*, 1982). A similar decrease in pulmonary inflammation has been observed in rabbits, guinea pigs, and mice subjected to repeated exposures to *Micropolyspora faeni*, the agent that causes farmer's lung disease in humans (Schuyler *et al.*, 1983,1987,1992), and *Thermoactinomycetes vulgaris*, which causes humidifier lung (Takizawa *et al.*, 1989).

The decrease in pulmonary inflammation during continued challenge has been attributed to desensitization, defined as suppression of preexisting DTH by administration of homologous antigen. However, desensitization is clearly not present in the above models of lung disease, because lymphocyte proliferation and antibody responses were not depressed. Since desensitization in other systems can be achieved by administration of an antigen by an unusual route, such

as orally (Weigle, 1973), it is rather surprising that immunologic desensitization was not evident in models of repetitive pulmonary instillation of antigen. Two other possible mechanisms, increased degradation of inhaled antigen or increased suppression of lymphocyte proliferation by AM, were not present in these models (Schuyler and Schmitt, 1985; Kopp *et al.*, 1988). Additional possibilities, including decreased exposure of the lung to antigen due to changes of clearance mechanisms or change of T lymphocyte subtypes, were not investigated.

B. Immune Complex-Mediated Lung Injury

Ward and colleagues extended these studies on immune complex-mediated lung injury using intratracheal instillation of antibody to bovine serum albumin (BSA), followed by iv administration of BSA. Lung injury was measured using morphology, leakage of labeled intravascular protein and red blood cells into the lung, and quantitation of the neutrophil enzyme, myeloperoxidase, in the lung (Johnson and Ward, 1974). Marked differences were shown in responses to instilled IgG versus IgA immune complexes.

IgG immune complex-mediated damage was characterized by neutrophil infiltration into the lung and evidence of increased pulmonary vascular permeability. It was neutrophil dependent (Warren *et al.*, 1991) and required IL-1β and platelet-activating factor (Warren, 1992), which were likely produced by TNFα-stimulated macrophages (Warren *et al.*, 1990). Expression of VLA-4 and CD18 (Mulligan *et al.*, 1993b,c), functioning CR1 receptors (Mulligan *et al.*, 1992a), and CD11a (but not CD11b), and ICAM-1 expression, were also involved (Mulligan *et al.*, 1993c). There was upregulation by ELAM-1 on pulmonary venules and capillary endothelium, perhaps mediated through a rat analog to IL-8 (Mulligan *et al.*, 1991,1993a), and upregulation of ICAM-1 expression modulated through TNFα (Mulligan *et al.*, 1993a).

In contrast, IgA immune complex lung injury was characterized by accumulation of mononuclear cells (Warren *et al.*, 1991), perhaps mediated through monocyte chemoattractant protein 1 (MCP-1) (Jones *et al.*, 1992). The injury was neutrophil and TNFα independent and was not modulated by increases of ELAM-1 expression (Mulligan *et al.*, 1992c) or TNFα secretion, despite a TNFα-induced increase of ELAM-1 expression. Iga-mediated injury was similar to IgG immune complex injury in that it was VLA-4, CD18, and ICAM-1 dependent, but was dissimilar in that it is more dependent on CD11b than CD11a expression (Mulligan *et al.*, 1993d). IgA immune complex lung injury was apparently mediated through nitric oxide or its derivatives (Mulligan *et al.*, 1992c). IL-4 and IL-10 protected against the pulmonary

response to IgG immune complexes, whereas only IL-10 protected against IgA immune complex-mediated injury (Mulligan *et al.*, 1993e).

C. Asthma Models

There are multiple animal (e.g., primate, sheep, guinea pig, dog, rabbit, rat) models of asthma which have been used to explore pathophysiologic aspects of asthma (Wegner *et al.*, 1991; Abraham, W; Murray *et al.*, 1991; Lukacs *et al.*, 1994b; Yamaya *et al.*, 1990; Du *et al.*, 1992; Waserman *et al.*, 1992; Coyle *et al.*, 1990) and as a method to test the effectiveness of various therapeutic agents. Although all have some resemblance to human asthma, there are substantial differences, especially in regard to the physiologic response to airway challenge and in methods to induce immune hyperreactivity. Typical protocols use intraperitoneal administration of antigen with aluminum hydroxide and/or Bordetella adjuvants.

Guinea pigs immunized intraperitoneally with OVA in aluminum hydroxide adjuvant, often with the addition of pertussis (Handley *et al.*, 1992; Mauser *et al.*, 1993), can be induced to form IgE and IgG_1 antibodies, exhibit airway and blood eosinophilia, and display early and late-phase bronchoconstriction (Cerasoli *et al.*, 1991) when reexposed to OVA. These animals also demonstrate increased bronchial reactivity to histamine or acetylcholine administered iv. Using this method of sensitization, guinea pigs exhibit strain differences in blood eosoniphilia and bronchial hyperreactivity (Winthereik *et al.*, 1992). Increased responsiveness to acetylcholine after antigen challenge and late-phase bronchoconstriction correlates with BAL neutrophilia (Asano *et al.*, 1994). This method of immunization is very different from that which occurs in humans, and the pulmonary physiologic response of guinea pigs is dissimilar to human asthma. Guinea pigs exposed to various parasite antigens also exhibit airway hyperreactivity (Yamaya *et al.*, 1990).

Nonhuman primates exposed to *Ascaris suum* antigen via the airway exhibit immediate skin test reactivity and either early or both early and late increases in airway resistance (Patterson and Harris, 1992). Dual responses are associated with more BAL eosinophils and a greater increase of BAL neutrophils (Gundel *et al.*, 1992).

Hirshman and colleagues found that *Ascaris*-sensitized basenji greyhound dogs exhibited a number of changes similar to those in humans with asthma including increased specific and nonspecific airway reactivity and increased numbers of BAL mast cells (Hirshman *et al.*, 1980, 1986). Spontaneous and induced histamine release from BAL mast cells was increased compared to control animals (Hirshman *et al.*,

1988). Tracheal muscle from these animals exhibited impairment of the usual increase in cyclic AMP in response to isoproterenol (Emala *et al.*, 1993), and coincident with the measurement of collateral airway resistance, high resolution CT scanning detected airway narrowing (Herold *et al.*, 1991; Corddry *et al.*, 1991). Chronic treatment with methylprednisolone decreased nonspecific airway reactivity and BAL eosinophil number (Darowski *et al.*, 1989).

Others have induced an asthma-like syndrome with increased bronchial reactivity and reaginic antibody in dogs immunized intraperitoneally as puppies with hapten–carrier complexes in aluminum hydroxide adjuvant (Kepon *et al.*, 1977). Later work extended this model by using ragweed antigen (Baldwin and Becker, 1993; Becker *et al.*, 1989). Immunized dogs exhibited immediate- and late-phase skin test reactivity (Becker *et al.*, 1988), increased antigen-specific and nonspecific bronchial reactivity, and increased BAL mast cells, eosinophils, and histamine (Becker *et al.*, 1989).

T cells appear to be important in animal models of asthma. Frew and colleagues demonstrated a substantial influx of non-CD8 (presumably $CD4^+$) T cells into bronchial wall mucosa and adventitia of aerosol antigen challenged guinea pigs undergoing late-phase bronchoconstriction (Frew *et al.*, 1990). Using picryl chloride epicutaneous sensitization, mice challenged with intranasal hapten exhibited peribronchiolar cellular infiltration and increased pulmonary resistance *in vivo* (Garssen *et al.*, 1991). Hypersensitivity to carbacol was present in tracheas from such animals and could be transferred with T cells from sensitized animals. This phenomenon could not be produced in athymic mice (Garssen *et al.*, 1991). Cyclosporin A and FK506 administration prevented the development of both the late asthmatic response and bronchial hyperresponsiveness after antigen challenge (Fukuda *et al.*, 1991). IL-4-deficient and class II MHC-deficient mice which lack mature $CD4^+$ T cells could not express peribronchiolar inflammation or BAL lymphocytosis and eosinophilia when exposed to OVA (Brusselle *et al.*, 1994). Exposure of mice to certain parasites, e.g., *Schistosomes*, also caused the appearance of intrapulmonary and BAL eosinophilia via an IL-4-dependent mechanism (Lukacs *et al.*, 1994b), perhaps by altering the balance of Th2 versus Th1 T cell numbers in the lungs.

Antibody to IL-5 can ablate the eosinophilic airway response to OVA exposure in sensitized guinea pigs and can even block the OVA-induced increased sensitivity to substance P (Chand *et al.*, 1992; Mauser *et al.*, 1993). Eosinophil infiltration into the tracheas of sensitized mice after aerosol antigen challenge is dependent on CD4 cells and

IL-5 (Nakajima *et al.*, 1992) and can be blocked by inoculations of IFNγ (Iwamoto *et al.*, 1993).

Gelfand and colleagues (Renz *et al.*, 1992) developed a model of asthma in BALB/c mice (typically high IgE responders) induced by repetitive inhalation of OVA. These animals exhibited increased specific IgE production, increased sensitivity to iv methacholine, and evidence of sensitized cells in LALNs and spleen capable of producing specific IgE and IgG1. Isolated trachea from sensitized animals were hyperresponsive to electrical field stimulation. Specific IgE antibody and increased airway reactivity could be induced in naive recipients by transfer of sensitized cells from LALNs, but not spleen cells, followed by a single aerosol OVA exposure. Low-IgE responding SJL/L mice failed to develop either IgE antibodies or increased bronchial responsiveness, although they developed specific IgG antibodies (Larsen and Wicht, 1962). Local airway challenge, as well as systemic sensitization, was required for the development of airway hyperreactivity (Saloga *et al.*, 1994). This suggested that local factors in addition to systemic sensitization were required for bronchial hyperreactivity. In this model, IFNγ administration during OVA sensitization both decreased specific IgE production and ablated increased airway reactivity. The effect of IFNγ was dependent on the route of administration. Systemic administration decreased serum-specific IgE, but not LALN-specific IgE production. Perhaps most importantly, the OVA-induced increase of airway reactivity was ablated by airway, but not systemic, IFNγ administration (Lack *et al.*, 1994). These results were compatible with an IFNγ-induced shift from a predominant Th2 to a Thl T cell response and also gave evidence for compartmentalization of both systemic and airway immune responses. Thus, airway hyperresponsiveness correlated with LALN, but not systemic sensitization.

D. T Cell-Mediated Hypersensitivity

T cell-mediated inflammation in the lung can result in pulmonary fibrosis or hypersensitivity pneumonitis (HP).

1. *Hapten-Immune Model*

Exposure of mice to a hapten instilled into the lungs caused systemic sensitization as measured by ear swelling after reexposure to the sensitizing hapten (Stein-Streilein, 1983). Furthermore, following epicutaneous sensitization with lipophilic trinitrophenylchlorobenzene and an intratracheal challenge with the water-soluble hapten, trinitrophenyl, pulmonary fibrosis developed. Intratracheal rechallenge with an unrelated hapten (dinitrophenol) did not produce pulmonary fibrosis

(Stein-Streilein *et al.*, 1987). This model was similar to previous models of contact sensitivity using the skin for both sensitization and challenge and was consistent with a T cell-mediated DTH process (Polack, 1980). Different inbred mouse strains of animals exhibited coincidence of skin reactivity and the ability to develop pulmonary fibrosis (Kimura *et al.*, 1992). Induction of tolerance by injection of hapten-coupled splenocytes before sensitization depressed both the skin and the pulmonary responses following tracheal challenge (Kimura *et al.*, 1993). The inflammatory and fibrotic responses to intratracheal hapten challenge were transferred with immune lymphocytes, but not with immune serum. *In vivo* administration of anti-CD4 and anti-CD8 antibodies to sensitized mice prevented or ameliorated the inflammatory and fibrotic responses to tracheal challenge. The development of pulmonary fibrosis is associated with BAL IL-2 activity and lymphotoxin mRNA in BAL nonadherent cells (Garcia *et al.*, 1992). An increased ratio of procollagen type I:III mRNA in the fibroblasts from immunized, challenged animals developed, indicating that qualitative as well as quantitative collagen differences occurred (Stein-Streilein *et al.*, 1992).

2. *Bleomycin-Induced Pulmonary Fibrosis*

Bleomycin is a mixture of glycoproteins from *Streptomyces verticullus* used clinically for its antineoplastic properties, but which predictably causes pulmonary fibrosis. In a hamster model, intratracheal administration of a single dose of bleomycin to experimental animals caused pulmonary fibrosis which resembled clinical pulmonary interstitial fibrosis, albeit with some differences in the pattern of fibrosis from usual interstitial fibrosis (Snider *et al.*, 1978).

Evidence has accumulated that cytokines are important mediators in animal fibrosis models. Increased TGFβ (Khalil *et al.*, 1989), TNFα (Piguet *et al.*, 1989a), IL-1 and IL-6 (Jordana *et al.*, 1988), MCP-1 (Brieland *et al.*, 1993), and macrophage-derived growth factor for fibroblasts (Denholm and Phan, 1989) have been detected in lungs or pulmonary cells derived from animals exposed to bleomycin. Administration of anti-TNFα can prevent fibrosis (Piguet *et al.*, 1989a).

Although many of the cytokine studies concentrated on the role of AM, recent reports indicate that pulmonary endothelial cells constitutively produced IL-6 which is increased by exposure to bleomycin (Karmiol *et al.*, 1993). MCP-1 is produced by fibroblasts (Rolfe *et al.*, 1992) and TGFβ is produced by pulmonary artery endothelial cells (Phan *et al.*, 1991,1992) or lung fibroblasts (Breen *et al.*, 1992). Therefore, the source of cytokines in bleomycin-induced pulmonary fibrosis

could include nonmacrophage pulmonary cells as well as macrophages.

Despite the evidence of toxicity of bleomycin-induced macrophage-derived cytokines, several studies have suggested that T cells may also be important. Different strains of mice responded differently to intratracheal bleomycin (Schrier *et al.*, 1983a). Pulmonary fibrosis did not occur in athymic nude mice (Schrier *et al.*, 1983b) and depletion of both CD4 and CD8 T cells prevented fibrosis (Jordana *et al.*, 1988).

3. *Experimental Hypersensitivity Pneumonitis*

Cormier and colleagues described a model of HP associated with alveolitis and fibrosis, and which was caused by repeated pulmonary instillation of *M. faeni*. It was associated with increased BAL IL-1α, IL-6, and TNFα (Denis *et al.*, 1991). Cyclosporin A administration blocked pulmonary fibrosis, but not alveolitis, and BAL IL-1α and TNFα, but not IL-6 (Denis *et al.*, 1992a), which suggested a role for T cells in producing fibrosis. Pulmonary fibrosis was also prevented by anti-TNFα antibody (Denis *et al.*, 1991). Evidence was also found for a role for AM-secreted TGFβ in promoting fibrosis (Denis and Ghadirian, 1992a). Secretion of TNFα, which probably originated from pulmonary macrophages, was fostered by CSF-1 and GM-CSF secreted by lymphocytes exposed to *M. faeni* (Denis and Ghadirian, 1992b). In contrast to an adoptive transfer model of HP to be described, *in vivo* depletion of T cells did not substantially affect the pulmonary histologic response to *M. faeni* (Denis *et al.*, 1992b). This might be related to repetitive challenges with an agent which has adjuvant effects (Bice *et al.*, 1974), so that inflammation in this model was macrophage, rather than lymphocyte, driven. Pulmonary fibrosis induced by repeated challenges with *M. faeni*, but not an increase of BAL inflammatory cells, was reduced by administration of anti-CD11a, implicating integrins in the processes that lead to fibrosis in this model (Denis and Bisson, 1994).

Lymphocytes can be implicated in other models of HP. Cyclosporin A administration ameliorated pulmonary lesions in animals subjected to airway challenges with *T. vulgaris* (Takizawa *et al.*, 1988). Nude mice did not exhibit pulmonary lesions of HP after exposure which was able to produce lesions in T cell-sufficient littermates. The ability to express pulmonary lesions could be transferred with T cells from sensitized mice (Takizawa *et al.*, 1992).

Schuyler and colleagues have developed an adoptive transfer murine model of experimental HP using *M. faeni*. Cells for adoptive transfer were obtained from spleen, peripheral lymph nodes, LALNs,

and peritoneal exudate from immunized animals. The cells were restimulated in culture with relevant antigen and could then transfer to naive recipients a susceptibility for increased lung inflammation following an intratracheal rechallenge with antigen (Schuyler *et al.*, 1991). IFNr and IL-2 were present in substantial quantities in cultures (Fei *et al.*, 1993), and CD4 cells were required at the beginning of culture to generate effective cells for the adoptive transfer (Schuyler *et al.*, 1994). The transferred cells were a mixture of naive and memory CD4 T cells, as defined by CD44, CD45RB, and L-selectin expression (Schuyler *et al.*, 1994a,1992). Successful transfer was also dependent on the presence of CD4 T cells in the recipient (Schuyler *et al.*, 1994b), suggesting the necessity of an important interaction between host and recipient CD4 T cells.

VI. Models for Lung Transplantation and Graft versus Host Disease

A. Lung Allograft Rejection and Graft versus Host Disease (GVHD) in the Lung: A Pathologic Comparison

Despite different methods to develop models for lung allografting or GVHD, the immunologic responses in the lung show similar histologic patterns. Several studies have documented these similarities (Atkinson *et al.*, 1971; Emeson *et al.*, 1982; Piguet *et al.*, 1989b; Randhawa and Yousen, 1992; Stein-Streilein *et al.*, 1981; Yousem *et al.*, 1990). The histoincompatible allografted lung is recognized as "foreign" by the recipient and, therefore, is subject to rejection (Randhawa and Yousem, 1992). In GVHD, donor immunocompetent allogenic cells recognize the recipient as foreign (Farrara and Deeg, 1991).

In both of these disorders, pulmonary manifestations of lung allograft rejection and GVHD may be separated into acute and chronic changes. In acute lung allograft rejection, the initial pathologic lesions of perivascular mononuclear cell infiltrates is termed "minimal rejection" or grade 1 (Yousem *et al.*, 1990). A more severe perivascular mononuclear cell infiltrate consisting of activated lymphocytes, plasma cells, and macrophages is called "mild acute rejection" or grade 2 (Yousem *et al.*, 1990). In some instances rare eosinophils are present (Yousem *et al.*, 1990). Vascular changes may include degeneration of the endothelium (endothelialitis) (Yousem *et al.*, 1990; Randhawa and Yousem, 1992), and lymphocytic infiltration of the bronchioles may be present (Yousem *et al.*, 1990). In grade 3 acute lung cancer allograft rejection, also known as moderate acute rejection, infiltrates progress and become more apparent around pulmonary veins, arterioles, and peribron-

chiolar areas (Yousem *et al.*, 1990). In grade 4 or "severe acute rejection" mononuclear cell infiltrates extend into air spaces and involve vessels and bronchioles. Necrotizing vasculitis and parenchymal necrosis may also be visible (Randhawa and Yousem, 1992).

In contrast to acute lung allograft rejection, the pulmonary pathology of acute GVHD has not been assigned histologic grades. Beschorner *et al.* (1978) first described the acute changes of GVHD in the lung in recipients of bone marrow transplants. In these patients, the pathology was limited to that of lymphocytic bronchitis (Beschorner *et al.*, 1978). More recently, Atkinson *et al.* (1991) reported an acute pulmonary syndrome after bone marrow transplantation that resembled acute GVHD of the lung. The lesions included lymphocytic peribronchial infiltrates, bronchial epithelial degeneration, and lymphocytic perivascular infiltrates (Atkinson *et al.*, 1991). Relative to the histology of acute lung allograft rejection, the pulmonary changes observed in acute GVHD of the lung are analogous to a grade 2 rejection response.

In animal models, investigators have reported acute "GVHD-like" changes in the lung (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981; Wilkes *et al.*, 1994a). In these studies the histologic lesions included alveolitis, lymphocytic bronchitis, and vasculitis. The histologic changes of acute GVHD were analogous to grade 4 or severe acute rejection in a lung allograft. In both allograft rejection and GVHD of the lung, the pathologic lesions of the airway began at the level of the bronchioles and extended into alveolar spaces.

The chronic stage of lung allograft rejection and GVHD is associated with the development of bronchiolitis obliterans (BO) (Farrara and Deeg, 1991; Rhandhawa and Yousem, 1992; Yousem *et al.*, 1990). Bronchiolitis obliterans is not a lesion specific to allograft rejection or GVHD and, in fact, has been associated with a variety of conditions (Epler, 1988) including toxic fume inhalation, rheumatoid arthritis, penicillamine use, postinfectious etiologies, as well as idiopathic causes. The histology of BO shows granulation tissue plugs within the lumens of the small airways, epithelial cell damage, mononuclear cell infiltrates, and, at times, complete obstruction of the airways (Epler, 1988; Randhawa and Yousem, 1992). Bronchiolitis obliterans observed in chronic lung allograft rejection and GVHD involve the membranous and respiratory bronchioles, and possibly involve more proximal airways (Randhawa and Yousem, 1992). In contrast to the pathology observed in the acute disease, lymphocytic perivascular infiltrates are present in only 40% of BO cases associated with chronic lung allograft rejection (Randhawa and Yousem, 1992). The degree of vascular involvement in BO associated with chronic GVHD is unknown but is likely less than that of acute GVHD.

B. Models of Lung Transplantation

Both canine and rat models have been utilized to study lung transplantation and the immunopathogenesis of rejection (Benfield, 1976; Prop *et al.*, 1985a,b). However, the availability of more immunological reagents has allowed the rat model developed by Marck, Prop, and Wildevuur to be more extensively studied (Marck *et al.*, 1983). Orthotopic transplantation of the Brown Norway rat lung allografts (RT^n) into Lewis (RT^1) rats resulted in histological and immunological changes analogous to that of human lung transplantation (Marck *et al.*, 1983; Prop *et al.*, 1985a,b). The rejection process occurred in four phases (Prop *et al.*, 1985a,b): (1) the latent phase which occurred immediately after transplantation in which no immunological activity was described in the graft (Day 1 after transplantation); (2) the vascular phase, characterized by infiltration of BALT and perivascular tissue by lymphocytes (Days 2 or 3 after transplantation); (3) the alveolar phase, with mononuclear cell infiltration of the alveolar walls (Days 4 or 5 after transplantation); and (4) the destruction phase, characterized by intraalveolar edema and destruction of airways and vessels by infiltrating mononuclear cells (Day 6 after post-transplantation). Significantly, these four phases resemble somewhat the four grades associated with acute rejection in humans (Yousem *et al.*, 1990).

While these studies described the histological changes associated with acute rejection, only two reports currently exist in the literature describing animal models of chronic rejection, known as BO (Hertz *et al.*, 1993; Uyama *et al.*, 1992). Utilizing the previously described rat lung allograft model, rats made tolerant to their allografts by cyclosporin developed the typical changes of BO around 6 months after transplantation. The airway lesions were associated with upregulated class II MHC expression on the epithelium in the large airways, aggregates of DCs in the submucosa, and ulcerated epithelium (Uyama *et al.*, 1992). In a murine model utilizing hetertopically transplanted airways, the characteristic lesions of BO developed in the allograft after 21 days (Hertz *et al.*, 1993).

A significant difference between the rat models and that of clinical transplantation is that only one or two doses of the immunosuppressant drug, cyclosporin, results in indefinite acceptance of the donor rat lung (Uyama *et al.*, 1992). Human lung allograft recipients usually require life-long therapy to prevent rejection (Trulock, 1993). However, without cyclosporin, the rat lung allograft undergoes a rapid rejection process which usually results in the destruction of the allograft in 7 or 8 days posttransplantation (Prop *et al.*, 1985a,b).

Although lung transplantation has become an increasingly utilized modality for the treatment of many endstage lung diseases (Trulock, 1993), the lung allograft, in both animal models and humans, is more prone to rejection than other solid organs (Prop *et al.*, 1985a,b; Trulock, 1993). The presence of many immunocompetent cells present in the donor lung that can stimulate a rejection response may be the explanation (Prop *et al.*, 1985a,b; Trulock, 1993). Notably, despite the large numbers of T lymphocytes present in the lung and thus carried into the recipient, the clinical syndrome of systemic GVHD has not been reported in human lung allografted individuals. However, GVHD in lung transplantation was reported in an animal model in which the recipient was rendered severely immunoincompetent by total body irradiation (Prop *et al.*, 1989).

Acute lung allograft rejection is believed to be initiated by donor lung APC, i.e., DCs and perhaps macrophages, interacting with recipient lymphocytes (Winter *et al.*, 1989). Although there is no direct evidence that these accessory cells mediate allograft rejection, several studies suggest their role in the rejection responses. Acute rejection episodes commonly occur at a time when there is an abundance of donor DCs and lung macrophages, i.e., the first 8 to 12 weeks after transplantation, and diminish when these cells are replaced by those of the recipient (Paradis *et al.*, 1985; Uyama *et al.*, 1993). Utilizing a murine model of renal transplantation in which DCs had been depleted, Lechler demonstrated that repletion of DCs resulted in the rejection of the allograft (Lechler and Batchelor, 1982). Similarly, blocking antibodies to DCs resulted in the prolongation of survival of murine pancreatic islet allografts (Faustman *et al.*, 1984). As discussed previously, DCs exist within the epithelium and subepithelial areas of the bronchi/bronchioles, areas that are involved in both acute and chronic rejection. Additionally, IFNγ, a cytokine crucial to the rejection process (O'Connell *et al.*, 1993), was shown to upregulate the number of DCs in the interstitium surrounding pulmonary capillaries, within the alveolar interstitium, and in the bronchial epithelium (Kradin *et al.*, 1991). Finally, DCs accumulate in areas of BO during the course of chronic allograft rejection (Uyama *et al.*, 1992). Collectively, these studies suggest a central role for DCs in the pathogenesis of lung allograft rejection. Lung macrophages, although suppressive of many immune cell functions, may also be involved in the initiation of the rejection response.

Lung accessory cell–lymphocyte interactions occur through cytokines and intercellular signals and result in upregulated cellular and humoral immunity (Wilkes and Weissler, 1994). Cellular immunity is

crucial in lung allograft rejection (Prop *et al.*, 1985a,b) and may result from the differential stimulation of Th1 versus Th2 cells. Th1 lymphocytes play a significant role in the pathogenesis of solid organ allograft rejection (Jordan *et al.*, 1991; O'Connell *et al.*, 1983). For example, in a murine example of pancreatic islet rejection, allograft infiltrating lymphocytes preferentially expressed mRNA for IL-2 and IFNγ, and not IL-4 (O'Connell *et al.*, 1983). Similarly, in rat lung allografts, IFNγ mRNA was expressed during the rejection episodes (Jordan *et al.*, 1991). The clinical importance of Th1 lymphocytes in allograft rejection is exemplified by the fact that the primary immunosuppressive agent used in recipients of human lung allografts is cyclosporin, which preferentially inhibits IL-2 and IFNγ production from lymphocytes (Cockfield *et al.*, 1993). In contrast, Th2 lymphocyte activity, i.e., production of IL-4 and IL-10, which downregulates Th1 activity, has been strongly associated with prevention of allograft rejection (Gorczynski and Wojcik, 1994). Both allogeneic AM and parenchymal lung DCs were potent inducers of IFNγ, but not IL-4, from lymphocytes (Wilkes and Weissler, 1994). Collectively, these data suggest that allograft rejection is in part mediated by lung macrophages and DCs stimulating Th1 lymphocytes.

Cytokines from Th1 and Th2 lymphocytes can both result in specific immunoglobulin production (Kitani and Strober, 1993). Therefore, the upregulated Th1 lymphocyte activity observed in allograft rejection might be responsible for the enhanced local immunoglobulin production observed during the rejection process (Wilkes *et al.*, 1994b). IFNγ, a Th1 cytokine, can stimulate IgG2a from murine B lymphocytes (Kitani and Strober, 1993). Furthermore, IFNγ production, induced by human lung macrophages, selectively stimulated IgG2 production from allogeneic peripheral blood mononuclear cells (Wilkes and Weissler, 1994). In recipients of lung allografts undergoing rejection, Wilkes (1995) demonstrated that local production of IgG2 was selectively upregulated and, thus, served as a marker for the rejection response (Wilkes *et al.*, 1994b). Few studies have demonstrated a role for alloantibodies in mediating the process of lung allograft rejection. Coronary atherosclerosis secondary to murine cardiac allograft rejection was in part mediated by antibodies directed against the donor coronary epithelium (Russell *et al.*, 1994). Similarly, IgG2, but not IgG1, IgG3, or IgG4, produced locally during human lung allograft rejection, preferentially bound to perivascular and peribronchial extracellular connective tissue matrices which are the anatomic locations involved in the rejection process (Wilkes, manuscript in preparation). Taken together,

these studies suggest a role for a Th1-dependent humoral responses in the pathogenesis of lung allograft rejection.

While lung accessory cell–T lymphocyte interactions initiate organ rejection (Winter *et al.*, 1989), the production of proinflammatory cytokines, IL-6 and TNFα, has been identified as a mediator of the rejection process (DeMeester *et al.*, 1993; Saito *et al.*, 1993). Evidence that TNFα was involved in rejection was demonstrated by DeMeester who reported that TNFα mRNA and protein were upregulated in lung tissue during acute rejection of rat lung allografts (DeMeester *et al.*, 1993). Significantly, anti-TNFα antibodies reduced the vasculitis and hemorrhagic lesions in rejecting lung allografts (Saito *et al.*, 1993). Similarly, IL-6 was upregulated in the lung during rejection and was postulated to be clinically important in following the activity of the rejection process (Rolfe *et al.*, 1993).

C. Models of Graft versus Host Disease in the Lung

GVHD is a systemic process, and relatively few have described pulmonary involvement in animal models of GVHD (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981). Stein-Streilein *et al.* (1981), utilizing a murine model, reported GVHD "reactions" in the lung. In these studies, suspensions of parental (MHA) lymph node cells were instilled into the trachea of F1 hybrid (MHA × CB) recipient hamsters. The histology observed in the recipient lungs showed mononuclear cell infiltration in the interstitium, alveolar, peribronchiolar, and perivascular areas. Some of the animals developed thymic atrophy and splenomegaly which suggested a systemic component to the disease process (Stein-Streilein *et al.*, 1981). Interestingly, when the cells were given iv or intracutaneously, the animals developed systemic GVHD without any distinctive pulmonary pathology (Stein-Streilein *et al.*, 1981).

Piguet also studied the pulmonary disease associated with GVHD. In these studies, irradiated F1 hybrid (CBA × B10) mice were injected with either parental T lymphocyte-depleted bone marrow cells or with parental bone marrow cells together with suspensions of lymph node cells as a source of T lymphocytes. In addition to the induction of systemic GVHD, the histology of the lung was similar to that reported by Stein-Streilein. Additionally, these investigators demonstrated the central role of T lymphocytes in the lung pathology of GVHD in that injection of T lymphocyte-depleted bone marrow cells did not induce pulmonary pathology (Piguet *et al.*, 1989b).

Wilkes *et al.* (1994a) recently reported that allogeneic (C57BL/6) BAL accessory cells (>90% macrophages), when instilled intratracheally into the lungs of normal BALB/c mice weekly for 4 weeks, induced a lymphocytic alveolitis, bronchitis, and vasculitis analogous to GVHD of the lung or acute lung allograft rejection. Unlike other animal models of GVHD (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981), these recipient mice had no evidence of systemic disease. Additionally, if no further allogeneic challenges were performed, the pulmonary lesions eventually healed.

As previously stated, the clinical manifestations of chronic GVHD in the lung are associated with the development of BO (Farrara and Deeg, 1991; Randhawa and Yousem, 1992; Yousem *et al.*, 1990). In contrast, no animal models of chronic GVHD have reported an association with this type of pulmonary disease.

The immunopathogenesis in animal models of GVHD has been well described (Antin and Farrara, 1992; Piguet *et al.*, 1989b). Similar to acute lung allograft rejection, acute GVHD has been associated with the production of several proinflammatory cytokines including TL-1, TNFα, and IL-6 (Antin and Farrara, 1992; Piguet *et al.*, 1989b). In fact, IL-1 receptor antagonist was shown to significantly inhibit GVHD (McCarthy *et al.*, 1991). Relative to the lung, anti-TNFα antibodies partially prevented the pulmonary pathology of GVHD (Piguet *et al.*, 1989b). While proinflammatory cytokines are involved in the pathogenesis of GVHD, T lymphocytes initiate the process (Antin and Farrara, 1992). Th1 lymphocytes are crucial in acute GVHD. In a murine model of GVHD, IFNγ and IL-2 were preferentially produced in the course of acute GVHD (Allen *et al.*, 1993) with similar findings to those reported by other investigators (Antin and Farrara, 1992). Therefore, the immune response to alloantigens in both lung allograft rejection and GVHD seems to be associated with upregulated Th1 lymphocyte activity.

A role of humoral immunity in GVHD has not been well defined. However, Wilkes *et al.* reported (1994a) that allogeneic BAL cells instilled into murine lungs resulted in the predominant local production of IgG2a. Additionally, only IgG2a was shown to be deposited in the perivascular and peribronchiolar extracellular connective tissues, the same anatomic locations involved in lung allograft rejection and GVHD of the lung. These data suggest that locally produced immunoglobulins recognize component(s) of the extracellular connective tissue matrix and may be involved in the pathogenesis of GVHD of the lung.

VII. Summary

No evidence has emerged which suggests that the principles of immunity derived from studies on cells from other body sites are contradicted in the lung and its associated lymphoid tissue. What is clear, however, is that the environment dictates the types of cells, their relationship to one another, and what perturbing events will set in motion either the development of an "active" immune response or tolerance. Investigating mechanisms for the development of lung immunity has increased our understanding of how human diseases develop and is continuing to suggest new ways to manipulate pulmonary immune responses. Demonstration that lung cells regulate both nonspecific inflammation and immunity through the expression of adhesion molecules and the secretion of cytokines offers hope for ways to design more effective vaccines, enhance microbial clearance in immunosuppressed hosts, and to suppress manifestations of immunologically mediated lung disease. Important lung diseases targeted for intensive research efforts in the immediate future are tuberculosis, asthma, and fibrotic lung disease. Perhaps even the common cold might be conquered. Considering the pace of current research on lung immunity, it may not be too ambitious to predict that these diseases may be conquered in the next decade.

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