Role of Complement, Chemokines, and Regulatory Cytokines in Acute Lung Injury

PETER A. WARD

Department of Pathology
University of Michigan Medical School
Ann Arbor, Michigan 48109

INTRODUCTION

Lung inflammatory injury triggered by intrapulmonary deposition of IgG immune complexes has proven to be an important model for developing an understanding of the role of various mediators in events that lead to tissue injury. A composite of events that are involved in pathophysiological processes causing tissue injury following intrapulmonary deposition of IgG immune complexes in rats is outlined in FIGURE 1. Initiation of this inflammatory reaction is accomplished by the airway instillation of rabbit polyclonal IgG antibody to bovine serum albumin (BSA), followed by the intravenous infusion of BSA. This results in deposition of immune complexes along the alveolar surfaces of lung. This event is, in turn, associated with activation of the complement system.[1] The composite result of these events is stimulation of pulmonary macrophages via a Mac-1-dependent (CD11b/CD18) pathway.[2] Stimulated pulmonary macrophages produce a series of cytokines, including the classical proinflammatory cytokines, tumor necrosis factor α (TNFα), and interleukin-1 (IL-1).[3] In addition, the chemokine macrophage inflammatory protein-1α (MIP-1α) has been demonstrated to be produced by these cells. Simultaneous production of IL-10 also occurs.[4,5] TNFα and IL-1 probably play their most important role by causing upregulation of vascular (endothelial) adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and E-selectin.[6] The simultaneous production of MIP-1α leads to another event, namely, autocrine stimulation of pulmonary macrophages, resulting in enhanced production of TNFα.[4] The upregulation of the vascular adhesion molecules in lung by TNFα and IL-1 leads to a sequence of adhesive interactions in which neutrophils first interact via their sialyl Lewis^x ligands with endothelial E-selectin,[7,8] which has been upregulated by TNFα and IL-1. In turn, adhesive interactions between neutrophil CD11a/CD18 (LFA-1) and endothelial ICAM-1 sets the stage for transmigration of neutrophils.[2,9] Neutrophils that become adherent to endothelial cells via these various molecules may also become stimulated by endothelial cell expression of platelet activating factor (PAF) and interleukin-8 (IL-8), for which there are high-affinity receptors on the surfaces of neutrophils. Neutrophils then commence to their migration into the alveolar compartment in a manner that requires platelet endothelial cell adhesion molecule-1 (PECAM-1).[10] Transmigration is presumably under the regulation of chemotactic factors. The arrival of neutrophils into the alveolar compartment, together with the presence of activated macrophages, leads to injury of lung via the production by phagocytic cells of oxidants such as superoxide anion ($O_2\cdot$), H_2O_2 and nitric oxide ($\cdot NO$),

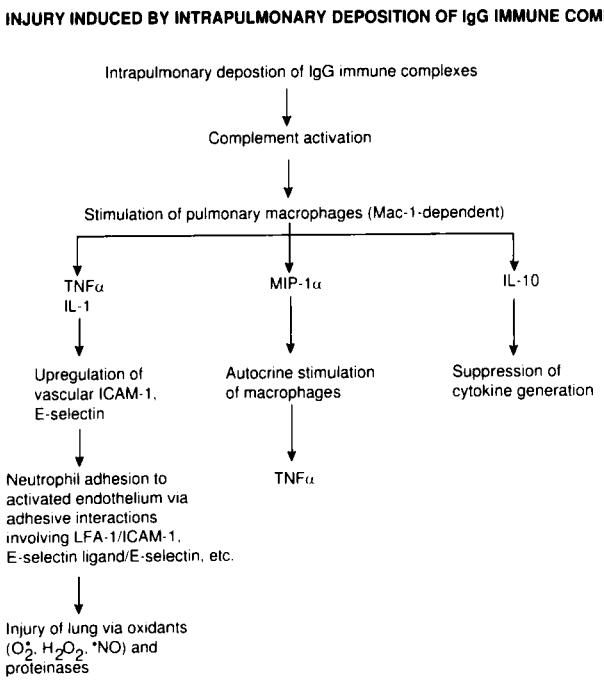

FIGURE 1. Role of cytokines in upregulation of endothelial adhesion molecules.

together with released proteinases including serine proteinases (e.g., elastase) and metalloproteinases (e.g., collagenase and cysteine proteinase).[11–13] As will be discussed below, ensuing production of IL-10 by lung macrophages in the course of this inflammatory model leads ultimately to suppression of cytokine generation, thereby diminishing any further increase in upregulation of vascular adhesion molecules, all of which causes a cessation of the inflammatory response.[14]

ROLE OF COMPLEMENT IN ADHESION MOLECULE EXPRESSION ON ENDOTHELIAL CELLS

It is well established that adhesion molecules play key roles in the recruitment of leukocytes into areas of inflammation. For instance, blocking of adhesion molecules, whether on leukocytes or on the endothelium, has been demonstrated to interfere with recruitment of leukocytes, (involving neutrophils, monocytes, and lymphocytes) (reviewed in reference 15). These findings imply that pathways that lead to upregulation of adhesion molecules will affect (enhance) leukocyte recruitment. Adhesion molecule upregulation on leukocytes chiefly involves CD11b/CD18 (Mac-1) on neutrophils and VLA-4 on mononuclear cells. Some of the most important aspects of the inflammatory response relate to upregulation of endothelial adhesion molecules. Stimulation of neutrophils by mediators such

as complement activation products (e.g., C5a) will cause granule fusion to the cell membranes resulting in increased expression of CD11b/CD18 (Mac-1) on the cell surface. Stimulation of leukocytes with a variety of mediators or with appropriate monoclonal antibodies can, in some cases, increase the binding affinities of $\beta 2$ integrins, which also implies more effective leukocyte adhesiveness to ICAM-1 of endothelial cells.[16,17] These observations suggest that leukocytes can be stimulated in a manner that more effectively permits engagement of their adhesion molecules with their "counter receptors" on endothelial cells. Ligand interaction with L-selectin on neutrophils also appears to cause signal transduction events within these cells, resulting in increased intracellular calcium.[18]

With respect to the endothelium (or monolayers of endothelial cells), activation events can result in enhanced expression of adhesion molecules. The abilities of TNFα and IL-1 to cause upregulation both *in vitro* and *in vivo* of endothelial ICAM-1 and E-selectin and the ability of IL-4 to cause upregulation of VCAM-1 are well documented. *In vivo* blocking of TNFα (by the use of antibody) in the IgG immune complex model of lung injury in rats significantly reduces *in vivo* upregulation of vascular ICAM-1 and, in turn, the recruitment of neutrophils.[3] Recently we have demonstrated that lung vascular ICAM-1 upregulation *in vivo* after intrapulmonary deposition of IgG immune complexes or after intratracheal instillation into rats of TNFα is nearly totally blocked under conditions of complement depletion (following serial intraperitoneal injections of purified cobra venom factor) or after complement blockade (with soluble complement receptor-1, sCR1).[19] This surprising observation implies a complement requirement for upregulation of ICAM-1, *in vivo*, an unexpected finding since TNFα alone is well able to cause the *in vitro* upregulation of ICAM-1 on endothelial cells. One possible explanation for the *in vivo* requirement for complement in upregulation *in vivo* of lung vascular ICAM-1 is described below.

It is becoming increasingly apparent that complement activation products can interact directly or indirectly with endothelial cells to bring about responses such as upregulation of adhesion molecules. For instance, C5a interacting with a relatively small number of binding sites on endothelial cells (approximately 1000 sites/cell) causes increased surface expression of P-selectin and secretion of von Willebrand factor.[20] As would be expected, these events are associated with increased neutrophil adherence to the endothelial cells in P-selectin-dependent manner. This increased adherence is independent of a requirement for either ICAM-1 or E-selectin. C5a des arg lacks the ability to activate endothelial cells. C5a also has other stimulating activities for endothelial cells, as reflected by its ability to induce increases in cytosolic $InsP_{1,4,5}$ as well as an increase in intracellular calcium.[21] Under these conditions, endothelial cells produce superoxide anion in a manner that apparently is dependent upon engagement of protein kinase C, inasmuch as inhibitors of PKC (such as staurosporin) are highly inhibitory. C5a-induced responses in endothelial cells are blocked if the cells are first incubated with pertussis toxin, suggesting a G-protein-sensitive pathway of signal transduction. Although up- or downregulation of C5a receptor on the endothelial cell has not yet been demonstrated, by reverse transcriptase polymerase chain reaction (RT-PCR) technology it has been shown that endothelial cells contain message for the C5a receptor.[20]

It is interesting to note that TNFα can also cause intracellular increases in calcium in endothelial cells, as well as superoxide anion production, but this pathway of activation is clearly different in that neither pretreatment of the cells with pertussis toxin nor with staurosporin interferes with the calcium increase or with superoxide production in TNFα-stimulated endothelial cells.[21] Accordingly,

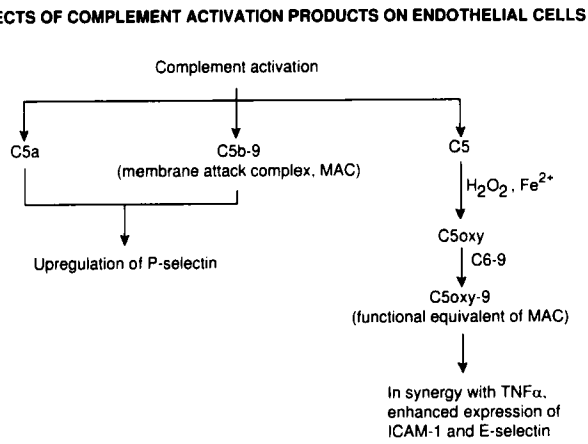

FIGURE 2. Generation of the functional equivalent of the complement-derived membrane attack complex by oxidation of C5 in the presence of iron, the product of which interacts with complement components C6–C9 to form the functional equivalent of the membrane attack complex.

the TNFα signal transduction pathway in endothelial cells is very different from that stimulated by C5a.

In addition to C5a being a powerful agonist for endothelial cells, the complement membrane attack complex (C5b-9) has also been implicated as an agonist, since it appears able to cause P-selectin expression on surfaces of endothelial cells. This causes increased adhesiveness for neutrophils in a manner that is blocked by antibody to P-selectin.[22] Recently it has been demonstrated that, if C5 is oxidized by hydrogen peroxide in the presence of reduced iron, an intact but altered C5 molecule acquires the functional activity of C5b, now being able to interact with C6-9 to form the functional equivalent of the membrane attack complex.[23] This membrane attack complex-like activity is termed C5oxy-9. When endothelial cells are treated with very small concentrations of TNFα in the presence of C5oxy-9, a greatly enhanced expression of both ICAM-1 and E-selectin occurs.[24] In turn, this is associated with increased adhesiveness of neutrophils to the treated endothelial cells in a manner that can be blocked by antibody to either ICAM-1 or E-selectin but not by antibody to P-selectin. Interestingly, C5oxy-9 does not, in and of itself, cause any change in the measurable levels of either ICAM-1 or E-selectin on endothelial cells. These data suggest that the membrane attack complex can function synergistically with TNFα to bring about enhanced upregulation of endothelial ICAM-1 and E-selectin. These data may explain the *in vivo* observations in which the unavailability of complement significantly reduces the ability of immune complexes deposited within lung to cause upregulation of lung vascular ICAM-1 and the subsequent influx of neutrophils.[19]

IN VIVO MEASUREMENT OF VASCULAR ADHESION MOLECULES

Measurement of lung adhesion molecules such as ICAM-1 is complicated by two problems. The first is that vascular ICAM-1 is constitutively expressed

in substantial amounts. In our recent studies in rats, it was apparent that 1.5 μg ^{125}I-anti-ICAM-1 vanished from circulation within minutes after infusion, due to binding with constitutive vascular ICAM-1.[19] This presents problems for *in vivo* measurement of ICAM-1. A second problem, at least in the case of lung, is the fact that type-II alveolar epithelial cells also constitutively express ICAM-1.[25] While it is assumed that alveolar epithelial cells cannot upregulate ICAM-1, this is based on *in vitro* observations and may not be reflective of *in vivo* behavior. Accordingly, measurement *in vivo* of ICAM-1, which is both constitutively expressed but also upregulated *in vivo*, requires caution. Because of dual sources of ICAM-1 in lung, immunochemical measurements of ICAM-1 in lung homogenates may not provide conclusive information about the level of vascular ICAM-1.

The most straightforward measurement *in vivo* of lung vascular ICAM-1 has been accomplished by the use of ^{125}I-anti-ICAM-1 (murine monoclonal antibody of the IgG_1 subclass).[19] The antibody (1 μg containing 1.5 μCi ^{125}I) is infused intravenously, together with 150 μg unlabeled anti-ICAM-1. Fifteen minutes later, rats are killed and the pulmonary vasculature infused with sterile saline in order to remove remaining blood. The amount of radioactivity remaining in lungs is then determined. In companion animals, the same amounts of ^{125}I-MOPC 21 (murine IgG_1) together with 150 μg unlabeled MOPC-21 are infused, using identical protocols. These animals provide information on the leakage of IgG_1 under conditions of evolving inflammatory injury. These radioactive counts are then subtracted from those obtained with ^{125}I-anti-ICAM-1 in order to correct the counts due to vascular leakage, allowing calculation of precise antibody fixation values. Under these conditions, it has been determined that there is approximately a 2-fold increase in lung vascular ICAM-1 4–6 h after intrapulmonary deposition of IgG immune complexes. This technique has allowed more precise determination regarding the extent to which upregulation of lung vascular ICAM-1 is dependent on cytokines such as TNFα. When rats undergoing intrapulmonary deposition of IgG immune complexes were pretreated with blocking antibody to TNFα, there was approximately a 70% reduction in upregulation of lung vascular ICAM-1, indicating that TNFα is directly involved in events *in vivo* leading to expression of ICAM-1.[19] As enzyme-linked immunosorbent assay (ELISA) technology becomes available, ICAM-1 levels in lung extracts can be directly determined, although for the reasons cited earlier interpretation of lung ICAM-1 may be subject to caution. The measurement of other vascular adhesion molecules such as E-selectin and VCAM-1, which are not constitutively expressed, should be more straightforward, although for such measurements recombinant rat adhesion molecules must be available.

ROLE OF ANTI-INFLAMMATORY CYTOKINES IN LUNG INJURY

Emerging over the past several years is the realization that a family of regulatory cytokines exists. These cytokines have anti-inflammatory effects that counter the proinflammatory effects of other cytokines, such as TNFα and IL-1. IL-4 and IL-10 have been earlier described to have suppressive effects on *in vitro* synthesis of TNFα, IL-1, and inducible nitric oxide synthase (iNOS).[26,27] We have demonstrated in the IgG immune complex model of lung injury that airway instillation of nanogram amounts of either murine recombinant IL-4 or IL-10 dramatically

reduces injury in a manner related to greatly diminished accumulation of neutrophils in lung. In turn, this has been attributed to >80% reduction in levels of TNFα recoverable from bronchoalveolar lavage (BAL) fluids. We have also shown that, in the case of IL-10, these protective effects can be directly related to virtually complete abrogation in upregulation of lung vascular ICAM-1.[14]

In more recent studies, the issue of whether intrinsic rat IL-10 functions as a regulator of IgG immune complex-induced lung injury has been addressed.[5] Rat IL-10 has been cloned and oligonucleotide probes have demonstrated that in lung tissue there is a progressive upregulation of mRNA for IL-10, with message appearing as early as 0.5 h, and peaking between 2 and 4 h after intrapulmonary deposition of IgG immune complexes. ELISA technology has confirmed that protein expression also occurs over a similar time-frame in this model. The next strategy was to block endogenous rat IL-10 by use of polyclonal antibody. When this was done, lung injury, as reflected by change in vascular permeability, increased (above positive reference controls) by 52%. In parallel, neutrophil accumulation in lung increased, as reflected either by myeloperoxidase (MPO) content or by BAL retrievable neutrophils, by 47% and 48%, respectively. Finally, and most importantly, the increased injury and neutrophil recruitment was reflected by a 56% increase in TNFα content in BAL fluids. Accordingly, these data suggest that, in this model of inflammatory lung injury, endogenous IL-10 is expressed and functions to regulate the intensity of the inflammatory response. Similar observations have been obtained with respect to IL-1 receptor antagonist (Shanley, T. P., and Ward, P. A., unpublished data).

ROLE OF MIP-1α CHEMOKINE IN INFLAMMATORY LUNG INJURY

Although the IL-8 family of chemokines is considered to be important in the chemotactic recruitment of leukocytes into inflammatory foci, the evidence is largely indirect, based either upon intradermal injection of chemokines or demonstration of mRNA and/or chemokine protein in tissues.[28] Recently, we have obtained direct evidence for the role of MIP-1α in the IgG immune complex model of lung injury.[4] Rat MIP-1α has been cloned and expressed and blocking antibodies obtained. Lung tissue extracts have revealed little mRNA for MIP-1α in lung tissue at time 0, but a progressive increase in message for MIP-1α occurring between 2 and 8 h. Protein expression in lung extracts followed a similar time course. When rats were pretreated with blocking antibody to MIP-1α, there was a 42% reduction in vascular permeability change, a 59% decrease in BAL neutrophils, and, most interestingly, a 45% reduction in TNFα levels in BAL fluids. These data indicate that, in the course of this lung inflammatory reaction, MIP-1α is upregulated and is required for the full development of neutrophil recruitment and lung injury. In companion studies using airway instillation of bacterial lipopolysaccharide into rat lungs, which induces a cytokine-dependent type of lung inflammatory injury, interventions similar to those previously described were employed. When MIP-1α was blocked systemically, lung vascular permeability fell 40%, BAL neutrophil counts fell by 92%, and BAL levels of TNFα fell by 67%.[4] Thus, in both models of inflammatory lung injury, MIP-1α is required and seems to be linked to TNFα production in lung. Although recombinant rat MIP-1α has chemotactic activity for both neutrophils as well as monocytes, the surprising finding was that BAL

TNFα levels fell significantly when MIP-1α was blocked. These data suggest that MIP-1α functions as an important autocrine stimulator of lung macrophages which, in these inflammatory models, are critical sources of TNFα and IL-1. The main function of TNFα and IL-1 seems to be upregulation of lung vascular ICAM and E-selectin, both of which are vitally involved in neutrophil recruitment in these models. To what extent MIP-1α also functions as a chemoattractant in these inflammatory models remains to be determined.

SUMMARY

The roles of complement, proinflammatory cytokines and regulatory cytokines in lung inflammatory injury are becoming defined. Like the proinflammatory cytokines (TNFα and IL-1), complement activation products (C5a and/or the membrane attack complex, C5b-9) can directly activate endothelial cells to cause upregulation of adhesion molecules (P-selectin) or can function in a synergistic manner with TNFα to cause enhanced upregulation of ICAM-1 and E-selectin. The β chemokine, MIP-1α, appears to function *in vivo* as an autocrine activator, enhancing TNFα production by pulmonary macrophages, which, in turn, enhances the inflammatory response. Finally, IL-4 and IL-10 have strong regulatory effects by suppressing *in vivo* production of TNFα. There is now compelling evidence to suggest that, in IgG immune-complex-induced lung inflammation in rats, endogenous IL-10 is produced and regulates the intensity of the inflammatory response. Blocking of endogenous IL-10 substantially increases lung TNFα production, the recruitment of neutrophils, and the intensity of lung inflammatory injury. Accordingly, the network of cytokines carefully regulates lung inflammatory responses.

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