Oxygen Radicals, Cytokines, Adhesion Molecules, and Lung Injury

Peter A. Ward

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

Inflammatory injury in the lung or dermis occurring after systemic activation of complement or after local deposition of immune complexes is related to local activation of tissue macrophages and/or recruitment of blood neutrophils. While requirements for cytokines (IL-1, TNFα, MCP-1) vary with the model of injury, requirements for adhesion molecules (β2 integrins, selectins, ICAM-1) differ. In most cases, the immediate events related to tissue injury can be linked to toxic products from oxygen and l-arginine. Whether there is a single toxic product or a variety of toxic products remains to be determined. These data emphasize similarities and differences in the mechanisms of inflammatory injury, as a function of the inciting inflammatory agent and the organ system involved. — Environ Health Perspect 102(Suppl 10):13–16 (1994)

Key words: lung, oxidants, neutrophils, macrophages, nitric oxide, complement, endothelial cells, cytokines, adhesion molecules

Introduction

Lung models of acute injury have been instructive about the complicated cascade of events leading to injury. In the models to be described, injury ensues as a result of recruitment and activation of inflammatory cells. In some cases, blood leukocytes represent the exclusive source of toxic products. In other situations, the toxic products are derived from a combination of resident phagocytic cells and recruited neutrophils. In yet another situation it appears that injury results exclusively from activation of resident phagocytic cells. The three models of acute lung injury (all in rats) are systemic activation of complement by cobra venom factor (CVF), intrapulmonary deposition of IgG immune complexes, and intrapulmonary deposition of IgA complexes. In all cases it appears that tissue injury is a result of oxidants generated from activated leukocytes and harmful effects of leukocyte-released proteases.

Lung Injury Produced by Systemic Activation of Complement

Intravascular (intravenous) infusion of the purified cobra venom factor (CVF) into rats causes massive, systemic (intravascular) activation of the alternative pathway of complement (1). Activation involving the third (C3) but not the fifth (C5) component of complement is insufficient to cause injury (2). After infusion of CVF and activation of complement, intravascular activation of complement occurs, resulting in stimulation of blood neutrophils. The features of CVC-induced lung injury are:

- Very rapid, early end point (30 min);
- Complement and neutrophil-dependent;
- Sensitive to catalase and deferoxamine;
- Independent of requirements for TNFα and IL-1;
- Dependent on CD11a/CD18 and CD11b/CD18 and ICAM-1;
- Dependent on P-selectin and L-selectin but not E-selectin;
- Damage occurring at endothelial interface with neutrophils.

The presumed sequence of pathophysiologic events is as follows: As neutrophils become activated, presumably by interaction of C5a with cell surface receptors on neutrophils, the cells undergo rapid homotypic aggregation, resulting in appearance of intravascular cellular aggregates. Simultaneously, contact with C5a leads to rapid upregulation of CD11b/CD18, the heterodimeric β2 integrin complex that is also known as Mac-1 (MO-1). Increased surface expression of CD11b/CD18 on the neutrophil sets the stage for eventual adhesive interaction with constitutively expressed endothelial ICAM-1, the "counter-receptor" for CD11b/CD18, as well as for CD11a/CD18 (LFA-1). The latter molecule is not upregulated during neutrophil activation. It appears that C5a as well as C3a cause release of histamine from rat mast cells and basophils. Histamine has the ability to react with receptors on endothelial cells, resulting in rapid translocation of P-selectin from Weible/Palade bodies to the surface of the endothelial cell (3). P-selectin is a transiently expressed glycoprotein of 140 kD whose counter-receptor is some type of glycoconjugate containing the oligosaccharide, sialyl Lewis`. It seems likely that neutrophil adhesive interactions with P-selectin expressed on endothelial cell surfaces, followed by endothelial ICAM-1 engagement with neutrophil LFA-1 and Mac-1, result in a firm adhesive interaction between the activated neutrophils and the adjacent vascular endothelial cells.

Lung vascular injury in this model occurs very rapidly, peaking within 30 min. It would not be expected that this would allow sufficient time for transcriptional and translational upregulation of the cytokines tumor necrosis factor alpha (TNFα) or interleukin-1 (IL-1); and, indeed, blocking of either cytokine fails to cause any reduction in injury (4). Damage of vascular endothelial cells is ascribable to toxic products generated from neutrophils that are in direct contact with endothelial cells (1). These products are derived from oxygen that is metabolized during the respiratory burst occurring in activated neutrophils. This is characterized by an abrupt increase in oxygen consumption and the formation of superoxide anion (O2−) and H2O2. Interception of either O2− (with superoxide dismutase [SOD]) or H2O2 (with catalase) will produce a substantial reduction in lung injury, as shown in Table 1. The protective effects of neutrophil or complement depletion procedures in this model of injury underscore the role of complement and neutrophil activation products in events leading to lung vascular injury. Pretreatment of animals with the iron chelator deferoxamine is also highly pro-

This paper was presented at the Conference on Oxygen Radicals and Lung Injury held 30 August-2 September 1993 in Morgantown, West Virginia.

The authors thank Ms. Shannon Grace for her excellent secretarial assistance. Address correspondence to Dr. Peter A. Ward, Department of Pathology, University of Michigan Medical School, MS240 Medical Science Building 1, 1301 Catherine Road, Ann Arbor, MI 48109-0802. Telephone (313) 763-6394. Fax (313) 763-4782.
Table 1. Protective interventions in CVF-induced lung injury.

| Intervention               | Reduction in lung injury, % |
|----------------------------|-----------------------------|
| Neutrophil depletion       | 72                          |
| Complement depletion       | 65                          |
| Catalase                   | 66                          |
| SOD                        | 53                          |
| Deferoxamine               | 74                          |
| Fe-deferoxamine            | 0                           |
| Anti-E-selectin            | < 5                         |
| Anti-L-selectin            | 50                          |
| Anti-P-selectin            | 47                          |

*As determined by use of E-selectin-Ig. Data from Till et al. (7), Mulligan et al. (4), and Ward et al. (5).

Table 2. Protective interventions in IgG immune complex-induced lung injury.

| Intervention               | Reduction in injury, % |
|----------------------------|------------------------|
| Neutrophil depletion       | 77                     |
| Complement depletion       | 62                     |
| Catalase                   | 75                     |
| SOD                        | 21                     |
| Deferoxamine               | 53                     |
| Fe-deferoxamine            | < 5                    |
| L-arginine                 | 55                     |
| Anti-E-selectin            | 95                     |
| Anti-L-selectin            | 33                     |
| Anti-P-selectin            | 0                      |

*Data from Mulligan et al. (7,8), Johnson and Ward (11,12), Ward et al. (13), Beckman et al. (14), Mulligan et al. (19), and Ward and Cochrane (20).

Intrapulmonary deposition of IgG immune complexes in rats is achieved by the airway instillation of rabbit polyclonal antibody to bovine serum albumin (BSA) and the intravenous infusion of BSA. Immune complex deposition develops along alveolar walls. The features of IgG immune complex-induced lung injury are as follows:

- Injury peaks at 4 hr;
- Complement and neutrophil-dependent;
- Requires toxic oxygen products of neutrophils and pulmonary macrophages (TNFα, IL-1);
- Sensitive to catalase and deferoxamine;
- Requires L-arginine;
- Dependent on CD11a/CD18 but not CD11b/CD18; requires ICAM-1;
- Requires E-selectin and L-selectin but not P-selectin;
- Extensive transmigration of neutrophils into alveolar compartment.

The outcome, as measured 4 hr later, is acute interstitial and intraalveolar edema and hemorrhage, attendant with large accumulations of neutrophils in the intraalveolar compartment (11). The neutrophil influx can be quantitated either by extraction of myeloperoxidase (MPO) from lung tissue or by bronchoalveolar lavage (BAL) and assessment of neutrophil numbers in the BAL fluids.

Injury in this model requires availability of both complement and neutrophils. Depletion of the former interferes with neutrophil influx into the alveolar compartment (11). Lack of availability of either complement or neutrophils causes a profound reduction (62–77%) (Table 2) in tissue injury. Toxic products of oxygen are also involved in events leading to injury. This has been determined by the protective effects of catalase (which destroys H₂O₂) or SOD, which has limited protective effects (21%) in this model of lung injury (12). Pretreatment of animals with the iron chelator deferoxamine leads to a 53% reduction in lung injury whereas the iron-saturated form of this chelator is without effect (13). These findings suggest the possibility of iron-dependent reduction of H₂O₂, which would result in production of long-lived species, although this is a presumptive conclusion. There is also evidence that lung injury related to this model can be drastically reduced in the presence of L-arginine analogues, such as N(ω)-monomethyl-L-arginine. The pathway of metabolism of L-arginine by nitric oxide synthase (NOS) appears to be:

L-arginine → NO (nitric oxide) and citrulline

NO + O₂ → ONOO⁻ (peroxynitrite anion)

ONOO⁻ + H⁺ → ONOOH → ·OH + NO₂⁻

While NO is relatively nonreactive, peroxynitrite anion (ONOO⁻) is highly reactive, as is ·OH (14). It seems that conversion of NO to ONOO⁻ is likely to occur especially when NO production results from activation of phagocytic cells in which a somewhat acidic environment would be present, as would be the case in an in vivo focus of inflammation. The cogenration of O₂⁻ (as well as H₂O₂) would lead to the interaction of ·OH with O₂⁻, and the subsequent biochemical steps described above. What cannot be accomplished at present is the demonstration of the precise relationship of L-arginine, as well as the cellular target of this factor. Although it has been shown that in murine macrophage-mediated killing of tumor cells in vitro that NO or its derivative interacts with aconitate, thus interrupting the electron transport chain in the Krebs cycle because of interaction with iron-sulfur centers in the enzyme, it is not clear if this can explain the effects observed in the lung injury model (156).

In the IgG immune complex model, the cytokines, substantial TNFα and IL-1, appear in BAL fluids obtained 3 and 4 hr after commencement of IgG immune complex deposition (16,17). These cytokines are functionally active, since their quantitation has been by employment of bioassays. Blocking of either cytokine leads to profound reduction in the neutrophil influx and reductions in the intensity of lung injury (16,17). The linkage between neutrophil influx and cytokine presence may be related to the ability of IL-1 and TNFα to cause vascular endothelial upregulation of the adhesion molecules E-selectin and ICAM-1. In view of the established requirement for ICAM-1 in this model, the requirements for β2 integrins are to be expected and have been demonstrated (18). What is unusual is the fact that neutrophil influx and accompanying lung injury in this model requires CD11a/CD18 but not CD11b/CD18. This is one of the
few situations in which dual requirements for both β2 integrins do not seem to persist. The use of blocking antibodies as well as Ig-selectin chimeric preparations has established that both E- and L-selectins are required for neutrophil influx and injury in this model (8,19). Blocking of these selectins reduces injury (and neutrophil accumulation) by 95 and 33%, respectively. Blocking of P-selectin is without effect, even though the same intervention is highly protective in the CVF model of acute lung injury. The ultimate injury occurring after intrapulmonary deposition of IgG immune complex involves toxic products from both oxygen as well as l-arginine, as described above.

### IgG Immune Complex-induced Dermal Vascular Injury

Studies companion to those described above in the IgG immune complex model of acute lung injury have been completed. Anti-BSA is injected intradermally, while BSA is infused intravenously. IgG immune complex deposition occurs primarily in venular walls in the dermis, resulting in complement activation and the influx of neutrophils (19,20). Depletion of either complement or neutrophils reduces injury by 65 to 68% (Table 3). One especially surprising finding has been that, while being highly protective against lung injury following deposition of the same IgG immune complexes, in the dermis the presence of catalase is without any protective effects, even though SOD is highly protective (21,22). This could suggest that dermal vascular endothelial cells are resistant to toxic oxygen products. Alternatively, the fact that SOD is highly protective in this model (reducing injury by 78%), and the finding that L-NMA is also significantly protective (23), could suggest that the chief agent of injury is related to interactions of NO with O2 to produce ONOO−, and ultimately, perhaps OH− (see above). The protective effects of deferoxamine might be related to a recent observation that this compound, being a potent iron chelator, can also react with ONOO− to bring about its chemical inactivation (24).

Recent studies have also demonstrated that IgG immune complex-induced dermal vasculitis requires IL-1 but not TNFα (25). This has been determined by the use of blocking antibodies, or by the use of TNFα receptor-1 or IL-1 receptor antagonist. Immunohistochemical studies reveal the appearance of IL-1, but not TNFα, in interstitial cells within the dermis following immune complex deposition (24). Why TNFα is not elaborated in the dermis under these conditions whereas in the lung there is abundant elaboration of both TNFα and IL-1 is unclear. Studies employing the intradermal coadministration of either TNFα or IL-1 with the anti-BSA have revealed that the recruitment of neutrophils and the expression of dermal vascular injury are both enhanced under such conditions (24). This demonstrates that the dermal vasculature is indeed reactive to both cytokines. Whatever the details, it is clear that the pathophysiology of dermal vascular injury is different when compared to similar injury occurring in the lung.

### IgA Immune Complex-induced Lung Injury

Features of IgA immune complex-induced lung injury are
- injury peaks at 4 hr;
- complement-dependent but neutrophil-independent;
- requires products of activated pulmonary macrophages;
- sensitive to catalase, SOD and deferoxamine;
- requires L-arginine;
- dependent on MCP-1 but independent of TNFα and IL-1;
- dependent on CD11a/CD18;
- independent of requirements for L-, E- and P-selectins;
- injury appears to be dependent on activated resident pulmonary macrophages.

Injury in this model is induced by the intratracheal instillation of murine monoclonal IgG1 anti-dinitrophenol (anti-DNP) followed by the intravenous infusion of DNP-BSA. Neutrophil depletion does not protect against injury, which is characterized by extensive injury of alveolar epithelial lining cells (25,26) (Table 4). Very few neutrophils appear in BAL fluids or in tissue sections of lung. The injury seems to involve the activation of pulmonary macrophages by IgA immune complexes and complement activation products. IgA immune complexes alone seem insufficient to cause macrophage-mediated injury. The complement requirement probably relates to the need for opsonization of complexes and the generation of complement activation products, the combination with complexes leading to macrophage activation. Pulmonary injury in this model is highly susceptible to the protective effects of catalase, SOD, and deferoxamine (Table 4). As expected, iron-saturated deferoxamine is without protective effects. There is compelling evidence in this model that toxic products from L-arginine play an important role, since the use of L-NMA leads to a high degree of protection (67%) (27). With respect to cytokine requirements, very little TNFα and IL-1 appear in the BAL fluids of these animals. Not surprisingly, anti-TNFα or anti-IL-1 do not reduce the intensity of injury following (28). In contrast, blocking of monocyte chemotactic protein (MCP-1) by antibody reduces IgG immune complex-induced lung injury significantly (approximately 80%) whereas this intervention in the IgG immune complex model of acute lung injury is without effect (29).

This sharply defines the requirement for cytokines in these two models of immune complex-induced lung injury. β2 integrin requirements in this model of injury demonstrate the involvement of both CD11a/CD18 and CD11b/CD18, and, as anticipated, the requirement for the "counter receptor" for these β2 integrins, ICAM-1 (27). Since there appears to be little if any need for the recruitment of blood leukocytes into the lung in this model of injury, it is not surprising that the process appears to be independent of a requirement for any of the three selectins (8). Taken together, these studies suggest that injury in this model is due to activation of residential intrapulmonary macrophages. Their activation probably requires involvement of CD11a/CD18 as well as CD11b/CD18.

### Table 3. Protective interventions in IgG immune complex-induced dermal vascular injury.

| Intervention  | Reduction in lung injury, % |
|---------------|----------------------------|
| Neutrophil depletion | 68 |
| Complement depletion | 65 |
| Catalase | 0 |
| SOD | 78 |
| Deferoxamine | 85 |
| L-NMA | 98 |

*Data from Johnson and Ward (21), McCormick et al. (22), Mulligan et al. (23), and Mulligan and Ward (24).*

### Table 4. Protective interventions in IgA immune complex-induced lung injury.

| Intervention  | Reduction in injury, % |
|---------------|------------------------|
| Neutrophil depletion | 76 |
| Complement depletion | <5 |
| Catalase | 95 |
| SOD | 100 |
| Deferoxamine | 77 |
| Fe-deferoxamine | 0 |
| L-NMA | 67 |
| Anti-E-selectin | 11 |
| Anti-L-selectin | 6 |
| Anti-P-selectin | 5 |

*Data from Johnson et al. (25), Warren et al. (26), Mulligan et al. (27,28), and Jones et al. (29).*
and lung injury is attributable to generation of toxic products from l-arginine as well as from oxygen. The demonstrated requirement in this model for ICAM-1 may be related to the anchoring of alveolar macrophages, via their β2 integrins, to ICAM-1 which is constitutively expressed on Type I alveolar epithelial cells.

Conclusion
Inflammatory injury as described in the various animal models is more complicated than first imagined. Differences have been found when comparing IgG immune complex-induced dermal vascular and lung injury and in the same organ (lung) when comparing two different stimuli (IgG versus IgA immune complexes). In part these differences can be related to the types of cytokines appearing in tissues after initiation of the inflammation of the inflammatory response. One of the more important in vivo functions of cytokines such as TNFα and IL-1 appears to be upregulation of vascular adhesion molecules. Another obvious pattern of differences is found in the role of the various adhesion molecules required for the full development of injury in these models. Why in the case of IgG immune complex-induced lung injury CD11a but not CD11b is important is unclear. The finding that appears to link all of these models, whether or not blood leukocyte recruitment is required, is the role of oxidants derived from oxygen as well as l-arginine. These toxic products appear to derive from phagocytic cells, either resident or recruited from the blood. A major problem at present is to define the patterns of differences (with respect to mediators required for injury) within the same organ and between organs, and to determine not only the chemical nature of the toxic oxidants but their targets as well. This information will assist in the development of therapeutic strategies designed to protect against harmful effects of the inflammatory response.

REFERENCES
1. Till GO, Johnson KJ, Kunkel R, Ward PA. Intravascular activation of complement and acute lung injury: dependency on neutrophils and toxic oxygen metabolites. J Clin Invest 69:1126–1135 (1982).
2. Till GO, Morganroth ML, Kunkel R, Ward PA. Activation of C5 by cobra venom factor is required in neutrophil-mediated lung injury in the rat. Am J Pathol 129:44–53 (1987).
3. McEver RP, Beckett JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. J Clin Invest 84:92–99 (1989).
4. Mulligan MS, Smith CW, Anderson DC, Todd RF III, Miyasaka M, Tamatani T, Issekutz TB, Ward PA. Role of leukocyte adhesion molecules in complement-induced lung injury. J Immunol 150:2401–2406 (1993).
5. Ward PA, Till GO, Kunkel R, Beauchamp C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J Clin Invest 72:789–801 (1983).
6. Mulligan MS, Polley MJ, Bayer RJ, Nunn MF, Paulson JC, Ward PA. Neutrophil-dependent acute lung injury: requirement for P-selectin (GMP-140). J Clin Invest 90:1600–1607 (1992).
7. Mulligan MS, Miyasaka M, Tamatani T, Jones ML, Ward PA. Requirements for L-selectin in neutrophil-mediated lung injury in rats. J Immunol 150:832–840 (1998).
8. Mulligan MS, Watson SR, Fennie C, Ward PA. Protective effects of selectin chimeras in neutrophil-mediated lung injury. J Immunol 151:6410–6417 (1993).
9. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69:927–938 (1992).
10. Mulligan MS, Paulson JC, De Frees S, Zheng Z-L, Lowe JB, Ward PA. Protective effects of oligosaccharides in P-selectin-dependent lung injury. Nature 364:149–151 (1993).
11. Johnson KJ, Ward PA. Acute immunologic pulmonary alveolitis. J Clin Invest 54:349–357 (1974).
12. Johnson KJ, Ward PA. Role of oxygen metabolites in immune complex injury of lung. J Immunol 126:2365–2369 (1981).
13. Ward PA, Warren JS, Till GO, Varani J, Johnson KJ. Modification of disease by preventing free radical formation: a new concept in pharmacological intervention. Baillieres Clin Haematol 2:391–402 (1989).
14. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624 (1990).
15. Hibbs JB Jr, Taintor RK, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated alveolar macrophage effector molecule (erratum 1989; 158:624). Biochem Biophys Res Commun 157:8–94 (1988).
16. Warren JS, Yabroff KR, Remick DG, Kunkel SL, Kunkel RG, Johnson KJ, Ward PA. Tumor necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. J Clin Invest 84:1873–1882 (1989).
17. Warren JS. Intrapulmonary interleukin-1 mediates acute immune complex alveolitis in the rat. Biochem Biophys Res Commun 175:604–610 (1991).
18. Mulligan MS, Wilson GP, Todd RF, Smith CW, Anderson DC, Varani J, Issekutz TB, Miyasaka M, Tamatani T, Rushe JR, Vaporsayan, Ward PA. Role of β1, β2 integrins and ICAM-1 in lung injury following deposition of IgG and IgA immune complexes. J Immunol 150:2407–2417 (1993).
19. Mulligan MS, Varani J, Dame MK, Lane CL, Smith CW, Anderson DC, Ward PA. Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. J Clin Invest 88:1396–1406 (1991).
20. Ward PA, Cochrane CG. Bound complement and immunologic injury of blood vessels. J Exp Med 121:215–234 (1965).
21. Johnson KJ, Ward PA. Biology of disease. Newer concepts in the pathogenesis of immune complex-induced tissue injury. Lab Invest 47:218–226 (1982).
22. McCormick JR, Harkin MM, Johnson KJ, Ward PA. Suppression by superoxide dismutase of immune complex-induced pulmonary alveolitis and dermal inflammation. Am J Pathol 102:55–61 (1981).
23. Mulligan MS, Hevel JM, Marletta MA, Ward PA. Tissue injury caused by deposition of immune complexes is l-arginine dependent. Proc Natl Acad Sci USA 88:6538–6542 (1991).
24. Mulligan MS, Ward PA. Immune complex-induced lung and dermal vascular injury. Differing requirements for tumor necrosis factor-alpha and IL-1. J Immunol 149:331–339 (1992).
25. Johnson KJ, Wilson BS, Till GO, Ward PA. Acute lung injury in rat caused by immunoglobulin A immune complexes. J Clin Invest 74:358–369 (1984).
26. Warren JS, Barton PA, Jones ML. Contrasting roles for tumor necrosis factor in the pathogenesis of IgA and IgG immune complex lung injury. Am J Pathol 138:581–590 (1991).
27. Mulligan MS, Warren JS, Smith CW, Anderson DC, Yeh CG, Rudolph AF, Ward PA. Lung injury after deposition of IgA immune complexes. Requirements for CD18 and δ1-arginine. J Immunol 148:3086–3092 (1992).
28. Mulligan MS, Vaporsayan AA, Miyasaka M, Tamatani T, Ward PA. Tumor necrosis factor alpha regulates in vivo intrapulmonary expression of ICAM-1. Am J Pathol 142:1739–1749 (1993).
29. Jones ML, Mulligan MS, Flory CM, Ward PA, Warren JS. Potential role of monocyte chemoattractant protein 1/JE in monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. J Immunol 149:2147–2154 (1992).