Mediator Gene Expression in Sepsis: Implications for Therapy

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Introduction

Diffuse lung injury resulting in the clinical entity commonly called the adult respiratory distress sydrome (ARDS), remains highly lethal. Since the clinical description of the syndrome about 20 years ago, mortality in published series of patients has changed very little. This is true in spite of major advances in the technology of critical care medicine. Although a large amount of research has dramatically enhanced the understanding of mechanisms of lung injury and respiratory failure in ARDS, this has, so far, not translated into demonstrably effective pharmacological interventions.

However, there are several pharmacological interventions currently being studied which hold promise. For example, a large multi-center prospective placebo controlled trial of the efficacy of the non-steroidal anti-inflammatory agent ibuprofen in patients with sepsis is currently underway and promises important new information about this class of drugs in this clinical setting. Preliminary clinical studies with the anti-oxidant n-acetylcysteine in patients with established ARDS and respiratory failure also appear promising. Conclusions about efficacy for this class of glutathione repleting drugs must await completion of definitive large studies. Other promising pharmacological interventions include a whole range of antioxidant substances, airway installation of surfactant, and manipulation of the generation or effects of various lipid mediators including platelet activating factor and eicosanoids. Recognition that diffuse lung injury resulting in ARDS is, at its root, an inflammatory condition has heavily influenced the development of therapeutic rationales.

The rapid development of techniques of molecular biology permitting manipulation of DNA has provided new opportunities for understanding the pathogenesis of diffuse lung injury and for developing novel therapies. Information describing the inflammatory process at the molecular level is directly applicable to many forms of diffuse lung injury. As alteration in expression of genes encoding important proteins in the process of injury is understood more thoroughly, new therapeutic rationales will emerge. For example, pharmacological interventions which have their effect as a result of altering gene expression may become useful. In addition, the new field of gene therapy may also be expanded to apply to acute diseases like ARDS.

This chapter will summarize some of the information relevant to altera-

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tions in gene expression which occur in the process of lung injury and how the techniques of molecular biology might be used in developing rationales for therapy and strategies for preventing or treating patients with diffuse lung injury. The chapter is not meant to be encyclopedic, but simply to illustrate the current advantages and potential for molecular biological methods in understanding and treating diffuse lung injury.

Experimental Endotoxemia as a Model of ARDS

Although ARDS occurs in humans in a number of clinical settings, in many reported series the predominant risk factor is sepsis [1,2,3]. As a result of that fact and of the fact that the pathophysiology of responses to endotoxemia in some experimental animals resembles the pathophysiology of ARDS, animal models of endotoxemia have been studied by numerous investigators interested in the clinical syndrome. For the past twenty years, we have used a chronically instrumented unanesthetized sheep endotoxemia model to study alterations in lung structure and function which we infer are relevant to the clinical syndrome of ARDS [4-7].

Fig. 1 schematically summarizes some of the pathophysiological responses to endotoxemia in chronically instrumented sheep. The response includes changes in function of both airways and the lung circulation. Alterations in lung mechanics include decreases in lung compliance and increases in resistance to airflow across the lungs. In addition to those acute changes in airway function, airway reactivity is also increased during the first few hours

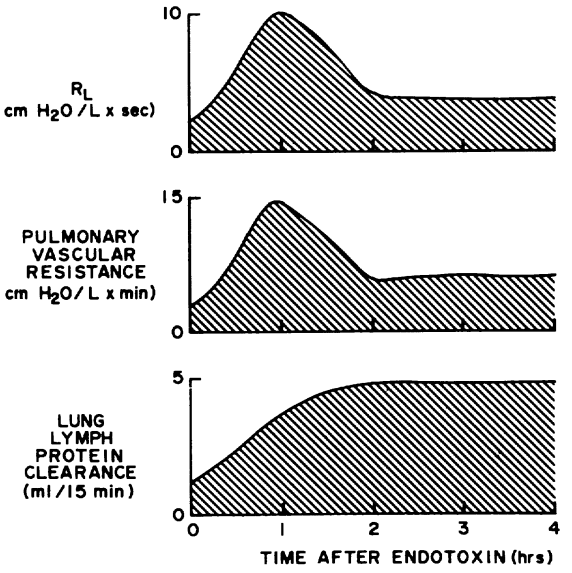

Fig. 1. Schematic representation of some changes in lung function caused by endotoxemia in sheep

following endotoxemia [8]. Pulmonary hypertension is a uniform and characteristic response to endotoxemia and is also common in patients with ARDS. In the sheep model, there is a marked early increase in pulmonary artery pressure and a more modest elevation in pulmonary vascular resistance which persists for several hours. The normal hypoxic vasoconstrictor response is lost following endotoxemia [9]. If sufficient doses of endotoxin are given to unanesthetized sheep, there evolves over 7 to 9 h a syndrome indistinguishable from the clinical syndrome of ARDS [10]. Animals develop severe respiratory failure, refractory hypoxemia, and diffuse pulmonary edema, and die of respiratory failure [10].

Bovine pulmonary artery endothelial cells in culture also are susceptible to toxic injury by low concentrations of endotoxin [11]. Since, in the whole animal, endothelial injury is an early and conspicuous event in the evolution of respiratory failure [12], endotoxin induced injury in cultured endothelial cells may have relevance to endotoxin induced injury of the whole lung.

Both the whole animal endotoxin preparation and cultured endothelial cell responses to endotoxin have been useful in deciphering pathogenetic mechanisms involved in acute lung injury. Information derived from a large body of research in these preparations now provides rationales for selection of pharmacological interventions which may be useful in humans.

Alterations in Gene Expression in Acute Lung Injury

Acute lung injury examplified by experimental responses to endotoxemia is a complexity of events. Release of numerous mediators, cytokines and other

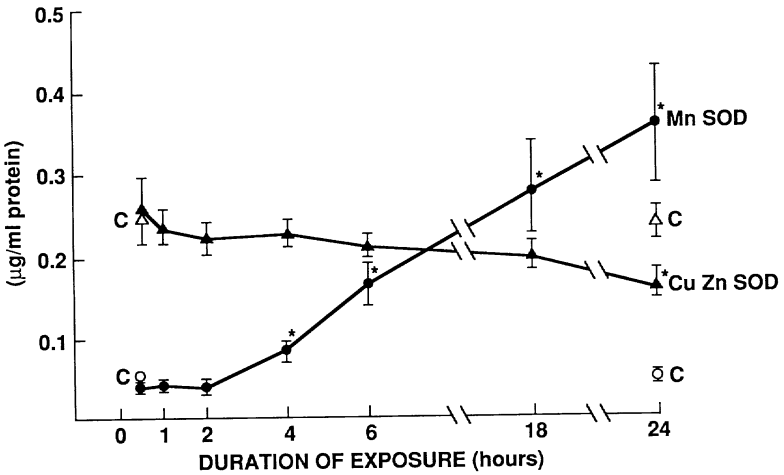

Fig. 2. Effects of endotoxin exposure on lung endothelial cell content of superoxide dismutase (SOD). (From [16] with permission)

chemicals which may participate in functional and structural derangements of the lung can be demonstrated. In addition, there is recent evidence that synthesis of several proteins which may directly or indirectly affect lung function is affected by endotoxin at a molecular level.

Several different kinds of data suggest that endotoxin induced injury of the lung involves, either directly or indirectly, oxidant stress [13-15]. Since oxidant stress might be thought to increase endogenous synthesis of antioxidant substances, we looked at the effects of endotoxin exposure of cultured bovine endothelial cells on endothelial cell content of several antioxidant enzymes. We found that endotoxin increased the amount of manganese superoxide dismutase (MnSOD) in a time course shown in Fig. 2 [16]. This increase in mitochondrial associated SOD was not accompanied by an increase in copper zinc SOD (the cytosolic form). In addition, as shown in Table 1, endotoxin exposure did not cause increases in lung endothelial cell content of catalase or of mitochondrial enzymes other than MnSOD [16]. In more recent studies, we have measured messenger RNA specific for MnSOD in the same endothelial cell cultured preparation, and have found that endotoxin exposure increases expression of the MnSOD gene (Meyrick, unpublished).

Numerous studies have demonstrated in both whole animals and in cultured cell preparations that endotoxin exposure causes release of several prostanoids [17-19]. In endothelial cells in culture, the increase in release of endothelial derived prostanoids (prostacyclin and PGE_2) appears to result, at least in part, from increased expression of the gene encoding the protein prostaglandin synthase. In our preliminary studies in both cultured endothelial cells and in the sheep endotoxin model, it appears that PGH synthase is induced as a consequence of endotoxin exposure, and that this molecular level event is important in the generation of prostanoids [20,21].

Table 1. Protein and enzyme concentrations in endothelial cells exposed to endotoxin. (From [16] with permission).

Time after endotoxin exposure, h	Protein	Glutathione Peroxidase	Catalase	Cyto-chrome-*c* oxidase	Fumarase
Control					
1	8.42±0.52	34±7	12±1	19±5	123±13
24	8.87±0.54	29±5	12±1	15±1	126±8
Endotoxin					
0.5	9.01±0.76	33±7	12±2	21±2	129±15
1	8.98±0.66	32±5	11±1	19±4	125±11
2	8.93±0.63	32±8	10±1	23±8	119±14
4	9.15±0.49	33±8	11±1	17±5	113±11
6	8.73±0.53	32±6	12±1	18±5	117±13
18	7.99±0.56	25±5	11±1	16±2	118±12
24	7.56±0.60	15±2	10±2	17±4	104±12

The endothelins are a family of peptides with potent biological properties. Endothelin 1 is a pulmonary vasoconstrictor which appears to be generated during inflammatory responses and, therefore, could participate in the pulmonary vasoconstriction typical of the endotoxin response. We looked at the time course of endothelial generation by the lung in sheep following endotoxin infusion and found increased production and release of endothelin in a manner consistent with increased expression of the gene encoding this peptide [22].

The macrophage derived cytokine, tumor necrosis factor (TNF), has been proposed as an important mediator of the physiological events of sepsis. We found that when TNF was infused into sheep, it was possible to reproduce many of the structural and functional features of the endotoxin response [23,24]. Endotoxin is the classic stimulus for generation of TNF by macrophages. The generation of TNF by macrophages following endotoxin exposure appears to involve increase in expression of the gene encoding the cytokine.

All of these data, and others, are consistent with the notion that endotoxin, a principal stimulus for acute lung injury resulting in ARDS in humans, induces increased expression of genes encoding a number of peptides which could be important either in the pathophysiology of respiratory failure, or in protecting the lung against extensive injury.

It is possible that a category of pharmacological interventions could be identified which would either exaggerate induction of genes encoding protective proteins, or repress induction of genes encoding destructive proteins. For example, in macrophages in culture, endotoxin induced increases in messenger RNA for TNF can be suppressed by the addition of prostaglandin E_2 to the cultures [25]. Since macrophages also generate prostaglandin E_2, it has been suggested that this may reflect a control system within the macrophage. In the sheep endotoxin model, we found that infusion of large amounts of prostaglandin E_2 significantly attenuated pulmonary responses to endotoxemia [26]. It is possible that this effect of prostaglandin E_2 is mediated via an effect on gene expression characteristic of the inflammatory response.

The Conventional View of Gene Therapy

As the techniques for analyzing and manipulating DNA have developed, a number of specific genetic abnormalities associated with specific clinical diseases have been identified. Classic examples related to the lungs would include alpha-1 antitrypsin deficiency and cystic fibrosis. Since in these cases, the disease appears to be a consequence of the lack of generation of a single normal protein, and the specific genetic abnormality is identified, it is presumed that the disease could be cured by introducing into the host genome a gene encoding the deficient protein which functions normally. A substantial body of work is now in progress in experimental preparations, and some preliminary work in humans is designed to test the notion that this is a

safe and effective rationale for treating diseases which are a consequence of a genetic abnormality.

Several strategies for delivering genes into intact organisms have been used. The most common strategy is to engineer cells in culture using retroviral vectors which assure insertion of the DNA into the genome of the cultured cells, and reimplanting these transformed cells into the intact organism. Such cells might correct the disease if engineered to hyperexpress a gene encoding a secreted protein deficient in the host. An example here in the lungs would be alpha-1 antitrypsin deficiency. Both genetically engineered fibroblasts and genetically engineered lymphocytes have been used in this manner.

For deficiencies of proteins which are either intracellular or integral to the cell, the problem is somewhat different. For example, the cystic fibrosis transmembrane regulator (CFTR) gene, the expression of which is abnormal in patients with cystic fibrosis, encodes a transmembrane protein which must be present in respiratory epithelium for ion transport to function normally. To effectively treat patients with cystic fibrosis, the CFTR gene would have to be expressed in a sufficient number of respiratory epithelial cells to correct the physiological abnormalities.

The goal for most of conventional gene therapy is to permanently alter host cells in a manner which assures that they will express the deficient gene, and will confer the ability to express the deficient gene to subsequent generations of cells. The goal here is a permanent alteration in the host genome.

Transient Gene Therapy (Gene Therapeutics)

Most diseases of the lungs, and other organs for that matter, are not, so far as we know, a consequence of a genetic abnormality. In the current context, ARDS is a transient disease. Patients are at risk, they either develop the syndrome or they do not, and they either live or die. As far as the pathogenesis of ARDS is understood, it is not a direct consequence of a genetic abnormality. However, if it were possible to transiently alter gene expression for proteins which were either protective or deleterious, then this might represent a novel therapeutic rationale.

Such approaches are at least theoretically possible. DNA which functions can be introduced into cells in a form in which it does not readily incorporate into the host genome. This is known in cultured cells as transient transfection. DNA in the form of plasmids which do not replicate in eukaryotic cells can be introduced by a variety of techniques into cultured cells and the encoding portions of the introduced DNA expressed in the host cells transiently. The potential for using this approach as a therapeutic modality, that is using DNA as a drug, has been termed transient gene therapy, or gene therapeutics.

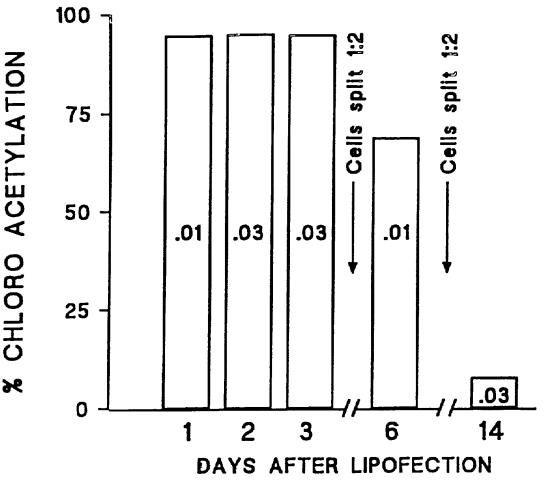

Fig. 3. Time course of expression of CAT activity in endothelial cells transfected with 10 μg DNA per 60-mm dish. All samples were incubated for 4 h in the CAT assays, and the numbers in the bars are milligrams of cell protein in the assay. The cells were split 1/2 on day 3 and day 6 after lipofection. CAT activity began to decline slightly after the first split of the cells and by 14 days after lipofection (8 days after the last split of the cells), was markedly reduced although still detectable. (From [29] with permission)

ARDS should be vulnerable to this approach. A principal focus of work in this area, presently, is the development of strategies for delivering genes into the cells of lungs of intact organisms.

Delivery of Genes to the Lungs

To date, two methods have been published for delivering genes directly into the lungs. Rosenfeld et al. [27] have used an adeno-virus vector delivered through the airways to express the human alpha-1 antitrypsin gene or the human CFTR gene in the lungs of cotton rats. It appears possible to obtain functioning cDNA in the lungs using this technique, although the quantitative generation of gene product may not yet have been demonstrated to reach therapeutic levels.

We have used small cationic unilamellar liposomes, originally described for use of transfection of cells in culture, and DNA in the form of plasmids to transiently express genes in the lungs of mice, rats, rabbits, and sheep [20,30,32].

Felgner et al. [28] originally described the synthesis of cationic liposomes which associated by charge with DNA and escorted DNA into cells in culture. Since that original description, several commercial preparations of these liposomes have become available. We initially tested whether lung endothe-

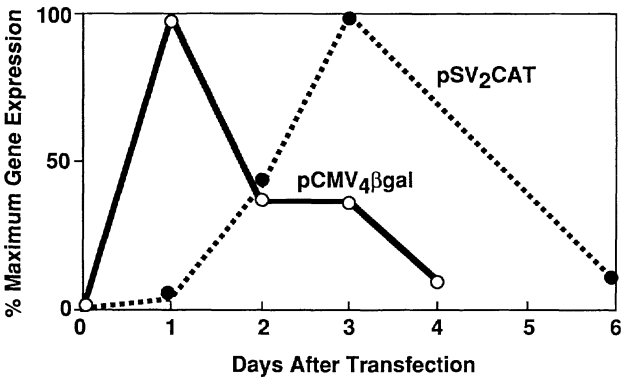

Fig. 4. Time course of expression of two different DNA constructs in transfected lung endothelial cells in culture

lial cells in culture would be vulnerable to transfection using this technique called lipofection [29]. As shown in Fig. 3, expression of the prokaryotic gene chloramphenicol acetyl transferase was achieved in lung endothelial cells in culture when a plasmid containing the cDNA for that gene driven by an RSV promote was introduced into the cells by lipofection. Expression of the gene was exuberant and lasted for days, even following splitting of the cells. Fig. 4 shows the time course of expression of the beta-glactosidase gene in a construct which the gene was driven by a CMV promoter, and the CAT gene driven by an RSV promoter. In both cases the cDNA for the reporter gene was contained in a plasmid and was introduced by lipofection in the cultured bovine lung endothelial cells. It is obvious that the method of lipofection works in lung endothelial cells in culture. The data suggests that the time course of expression may be manipulable by choosing the DNA construct. We tested whether the technique of lipofection could be used to transfect the lungs *in vivo* with foreign DNA. We reasoned that, since the lungs were the first microvascular bed seen by intravenously injected substances, intravenous injection of DNA liposome complexes might transfect endothelial cells in the lung. In addition, the lung is uniquely available by administering DNA liposome complexes through the airways. In the initial studies in mice reported in 1989 [30], we injected DNA liposome complexes either intravenously or intratracheally into mice. The DNA construct contained chloramphenicol acetyl transferase gene as a reporter driver by an SV-40 early promoter. The time course of expression of the CAT gene in the lungs of mice is shown in Fig. 5. The time course was similar to that which we saw in cultured endothelial cells. Expression of the foreign gene began at about day 1, peaked at about day 3, and persisted for several days thereafter. Table 2 summarizes the data using the CAT reporter gene in mice. From those data it

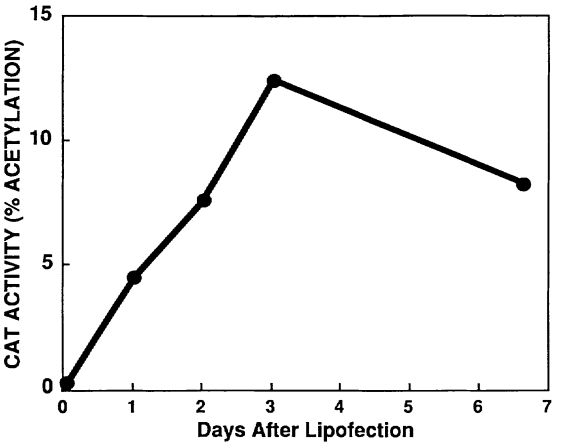

Fig. 5. Time course of expression of CAT gene in lungs of mice following intravenous lipofection

appeared that there was a dose related expression of DNA delivered by lipofection intravenously in the lungs. When material was injected via the airway, greater expression in the lung was seen for a similar dose of DNA. Intraperineal injection of the DNA liposome complexes resulted in no expression of the foreign gene. In those studies, we found expression of the gene limited to the lungs, that is we saw no detectable chloramphenicol acetyl transferase activity in either liver or kidneys regardless of the dose or route of DNA liposome injection.

We have now constructed additional vectors containing the cNDA for human alpha-1 antitrypsin driven by a CMV promoter. When this DNA construct complexed to small cationic liposomes is injected intravenously or delivered by aerosol to rabbits, expression of the gene can clearly be demon-

Table 2. CAT activity in lungs, liver and kidneys 72 hours following DNA-liposome injection into mice (from [30] with permission).

	% Chloramphenicol Acetylation per h per μg Protein X 10^{-2}		
Route	Lungs	Liver	Kidneys
Intravenous			
30 μg DNA per mouse	24.5	0	0
15 μg DNA per mouse	10.1	0	0
Intratracheal			
30 μg DNA per mouse	35.7	0	0
Intraperitoneal			
30 μg DNA per mouse	0	0	0

strated in the lungs. It appears that the alpha-1 antitrypsin gene product localizes to the epithelium in the airways regardless of the route by which the foreign DNA is delivered. Using a similar construct with the ovine PGH synthase gene in rabbits, we also have been able to show expression of the introduced CDNA in the lungs [31,32]. Recently, we have completed studies in sheep in which we have instilled the alpha-1 antitrypsin construct via bronchoscope into a lung sub-segment and subsequently demonstrated generation of human alpha-1 antitrypsin by the transfected segment removed from the animal (Canonico et al., unpublished).

All of these data clearly demonstrate the phenomenon that it is possible to deliver functioning foreign DNA into the lungs of intact animals. Using liposomes, the delivery procedure appears to be safe, at least acutely. Quantitative questions remain. It is not clear yet that sufficient generation of protein is achieved via this route to effect a therapeutic outcome.

What Genes Should be Manipulated to Treat ARDS?

As the delivery technologies are developed sufficiently to make this approach to gene therapy feasible clinically, further development of therapy will depend on the development of effective rationales for selecting genes, the expression of which should be altered to prevent or treat acute lung injury. In theory, it would be possible either to increase amounts of gene product or decrease them by introducing sense cDNA driven by a strong promoter, or anti-sense DNA driven by a strong promoter. There is good reason to think that expression of several genes could be altered in a way that could be beneficial to the lungs.

For example, as discussed above, oxidant stress appears to be an important common denominator for many types of acute lung injury. The endogenous proteins superoxide dismutase and catalase and other proteins are intimately involved in redox regulation in the lungs as in other organs. There is evidence that hyperexpression of manganese superoxide dismutase inside lung cells might protect them from oxidant stress. The human manganese SOD cDNA is available, and in theory could be delivered by the strategies discussed above in a manner which would enhance the resistance of the lungs to oxidant stress. Similar rationale could be used for a variety of other proteins including antiproteases (proteolytic injury may also be important in acute lung injury).

If foreign genes could be selectively introduced into sub-populations of lung cells, this might pave the way for gene therapeutics used in a manner to protect against pathological changes in lung function. For example, the endothelial derived prostanoids, prostaglandin E_2 and prostacyclin, have many effects which might be protective of the lung. Prostaglandin E_2 is generally anti-inflammatory, and prostacyclin is a pulmonary vasodilator. If the intravenous delivery of cDNA selectively transfected endothelial cells in the lungs, then the principal product of hyperexpression of prostaglandin syntha-

se would be expected to be prostaglandin E_2 and prostacyclin. Our early experimental data suggest this to be true [20].

If the generation of cytokines is principally a consequence of increases in gene expression, then it might be possible to manipulate production of cytokines using gene therapeutics. If TNF is a mediator of the pathology of endotoxemia for example, and anti-sense DNA for TNF were hyperexpressed in the cells which generate TNF, then it might be possible to diminish production of this toxic mediator over a transient period of time during which the lungs were at risk for injury.

It is obvious from this discussion that a large list of potential genes, either the hyperexpression or diminished expression of which might be beneficial to the lungs, could be generated.

Potential Problems

The principal problems currently in developing this technology for clinical application are the demonstration of safety and efficacy. It seems intuitively likely that the introduction of DNA in forms which do not readily incorporate into the host genome should be safer than altering the host genome with foreign DNA. However, introducing foreign DNA in any form into the cells of humans has long term consequences which remain unknown.

Most of the studies to date have failed to demonstrate high concentrations of gene product in the lungs regardless of the manner or route by which it is introduced. Obviously, efficacy will depend upon the quantitative generation of the protein encoded by the introduced gene. It is likely that this problem will be overcome, by the development of more efficient vectors and more efficient delivery systems for the lungs. However, it will be important to demonstrate efficacy in animal models prior to applying such technology in the clinical setting.

Conclusion

Current understanding of the pathogenesis of acute lung injury resulting in the clinical syndrome of ARDS provides a basis for multiple rationales for both pharmacological and other interventions. The development of the techniques of molecular biology now allows those investigating acute lung injury to understand the pathogenesis of the injury at a molecular level.This understanding will allow the design of pharmacological interventions which may affect gene expression as their principal mode of action.In addition, the development of strategies for delivering functioning DNA to the lungs in a form in which the DNA does not alter the host genome and is expressed only transiently, may allow gene therapy to be applied in this acute self-limited clinical situation. Initial studies in that regard appear promising.

It seems likely that the tools of molecular biology, over the years, will have a major impact on both the understanding and therapy of the processes involved in acute lung injury and the clinical syndrome of ARDS.

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