THE JANET FISCHER LECTURE: THE EFFECTS OF INHALED ENDOTOXIN, WHAT DO WE NEED TO LEARN?

Robert Burrell
West Virginia University (Ret.)

INTRODUCTION

The work that I have brought to the Cotton and Other Organic Dust Conferences since 1980 has been to see if I could bring points from the fields of medical microbiology and immunology to the problems following the inhalation of cotton dust. The question became: what contributions could contaminating microbial components present in these dusts make in leading to byssinosis or pre-byssinotic states?

1. The importance of endotoxin, a well known structural component of ubiquitous Gram negative bacteria, has been recognized for decades in a broad variety of pathogenic mechanisms including injury to the lung from human medicine. Of paramount importance are the matters of septic and endotoxin shock. In spite of modern medical intensive care procedures and in part, somewhat in thanks to them, the problem of adult respiratory distress syndrome remains important in morbidity and mortality in modern hospitals. The importance of endotoxin is also well known in such medical/surgical complications as disseminated intravascular coagulation and multiple organ system failure. Through the eighties, surgeons have been describing what they call post-surgical translocation, i.e., the release of endotoxin from the bowel during abdominal surgery. And we must not forget that an entire industry has developed around the preparation of pyrogen-free injectables wherein it has long been recognized that sub-nanogram levels of endotoxin contaminating fluids to be given intravenously can cause fever and inflammation in recipients.

The logical question then arose: "Is there any danger in inhaling endotoxin?" and the answer to that question, thanks to many of the participants in these conferences during the last twenty years has been an unqualified, "YES". TABLE I lists a number of environmental situations where airborne endotoxin has been associated with various human syndromes.

2. Endotoxin biologists now recognize that lipopolysaccharide or LPS, the major, purified component of endotoxin, is an extremely ancient enemy of all animals, vertebrate as well as invertebrate, and countless ways have evolved in recognizing this dangerous moiety. Table II lists the most well-described mechanisms the body has developed to recognize and interact with LPS. To this list, we should realize the body also has receptors for mannans and from the paper of Dr. Williams and associates presented earlier in this conference, it is clear that receptors are also present for beta 1,3-D-glucans.

3. Inasmuch as these various LPS receptors occur in tissues throughout the body, it is instructive to review those sites occurring in the respiratory apparatus as seen in Table III:

4. In my investigations of human respiratory diseases, I have always striven to set up models of human disease in experimental animals to approximate the human condition as much as possible. If the model could not approximate human exposure or produce similar human responses, it was scrapped. One of the most important ways of approximating the human condition was to pay particular attention to the matter of dose. One of the first dose considerations to ask is what would a realistic level of endotoxin be in the most extreme case of human/endotoxin interaction, i.e., that of endotoxic shock?

From the clinical literature, an AVERAGE level of circulating endotoxin in septic humans with clinical signs and symptoms has been reported to be 50 pg/ml. If we consider a standard 75 kg patient with a 6.0 L blood volume and make a rather large assumption that this endotoxin value reflects the same concentration in all tissues, it would be equivalent to a dose of about 40 ng/kg, a dose far below what many people use when working with experimental animals.

5. We should now compare this arbitrary, calculated dose with those obtained from human studies following intravenous challenge with purified LPS. The best studies have used standard doses of one of two well-described preparations, the American and the German standards. The characteristics of these two preparations are detailed in Table IV and further descriptions as well as the results obtained from the use of them may be found in reference 2.

It will be noted that although the origins and details of production of the two LPS preparations vary, the maximally-tolerated doses of both are the same, 1-4 ng/kg and it should be recalled that amount needed to present clinical signs and symptoms of endotoxic shock may be as little as only 40 ng/kg.

6. So what we first need to know in our experimental animal studies is how they will respond to comparable doses. Although we do not have data for inhalation doses comparable to the human IV studies, we ought to at least calculate a total bioburden of LPS administered to all of our animals.

Inhalation studies from our laboratory exposed hamsters for 5 hrs at 4 mg/cu m and one can calculate the total bioburden from the following equation:

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:149-152 (1997)
National Cotton Council, Memphis TN

Minute volume x Exposure time x [Aerosol] x % Resp. particles

In our hands aerosols are delivered with the DeVilbiss apparatus which has a published production efficiency of producing droplets in the 1-5 mm range at about 85.5%. Thus, we find that

0.00005 ml/min x 300 min x 4 mg/cu m x 0.855 = 0.0513 mg/ hamster
0.513 mg/kg

each animal exposed to a real-world concentration of 4 mg/cu m for the 5 hr duration experiences a total bioburden of 0.513 mg/kg.

Similarly, Rylander's group using Guinea pigs exposed to a 40 min duration of a more concentrated LPS administered by the Henderson apparatus and obtains:

0.0002 ml/min x 40 min x 100 mg/cu m x 0.70 = 0.56 mg/g. pig
1.6 mg/kg

a value of the same order of magnitude as that of our hamsters and although both values SEEM much higher than the human IV doses we must remember that we do not know how much of the inhaled doses actually escapes the respiratory compartment into the vascular system. This is a good area for investigation: how much of a respired dose of LPS enters the vascular system and at what dose? If we knew the answer to this, we would open a large literature to us from studies using intravenous challenges from which to make comparisons.

7. It would be instructive for endotoxin inhalation biologists to see what kinds of parameters have been used in these human IV challenge studies (see Table V). Although the results and significance of these parameters are discussed in detail in reference 2, some special points might be made with regards to presentations made at this year's conference.

Note that all of these parameters are objectively measurable. IV studies have shown that self-reported symptoms are unreliable. Not all of these values lead to useful information. The number of cells exhibiting a particular phenotype may not show much change, but if these same cells are tested for expression of cytokine production upon subsequent stimulation, significant alterations may be seen. IL-1 measurements have not been useful in that little change has been noted in post-challenge specimens. Further, the individuals show much more production of a molecule that competes for the same receptor, namely the IL-1 receptor antagonist, suggesting that the initial response may be anti-inflammatory rather than pro-inflammatory.

For those contemplating further human inhalation challenge, I would strongly urge that they would add some of these useful parameters to their protocols. By doing so it would enable inhalation biologists to tap into the far wider field of results from intravenous studies. A similar case could be made of making inhalation studies with the EC-5 preparation. It is known that significant changes occur in free lung cells of humans challenged with intravenous endotoxin (although their is little change in the numbers of BAL cells), but we have no information on changes taking place in human peripheral cells following LPS exposure.

8. A brief summary of the most significant human inhalation studies is given in TABLE VI and of course are mostly familiar to members of this conference. These and others are discussed in greater detail in reference 1.

9. Now of course not every one is interested in endotoxin. However, no one can afford to ignore since it is so ubiquitous and since such minute quantities are capable of exerting profound results, endotoxin contamination may lead to erroneous conclusions. One must take particular care to exclude contaminating endotoxin from studies. At the least one should show by means of Limulus tests that key reagents, media, etc. are endotoxin-free. Careful production of biological reagents and/or subsequent removal by endotoxin-binding ligands are essential and possible. A selection of examples is given in Table VII.

10. Endotoxin biologists are keenly aware that bacterial endotoxin is a profound immunomodulator and although these aspects have been reviewed (referencess 3 and 4), so much has happened since then that a modern update is sorely needed. It is important to differentiate classical antigen-specific immunity from the much older antigen non-specific type of immunity which figures greatly in responses to endotoxin (Table VIII).

For these purposes we shall define immunology as the study of altered host responses to stimulants or substances that are non-self. Immunology has classically concerned itself with recognizing non-self by antigen-specific or adaptive responses wherein primary encounter with an antigen induces specific response characteristics that are magnified after secondary encounter with that same antigen. Following secondary contact with antigen, specific immune responses are often qualitatively and quantitatively different. This immunologic memory (anamnestic response) is one of the most fundamental characteristics of the immune response. Measurement of antigen-specific responsiveness usually involves measurement of efferent responses, i.e., those dealing with changes in antigen-specific antibodies or T cells.

However, much of immunology is also concerned with non-antigen specific immunity in which recognition of non-self is achieved through biochemical means, i.e., via

evolved receptor molecules that recognize general classes of non-self molecules, e.g., polysaccharides. Innate immunity has evolved a limited repertoire of receptors that have been selected by evolutionary means to recognize certain foreign patterns and these receptors are not subject to somatic diversification. Measurement of non-antigen specific responses typically involve changes in afferent responses. Contemporary immunologists recognize that the innate, non-antigen dependent mechanisms may also be changed due to prior contact with a stimulant. Inflammatory cells become physiologically activated and primed by receptor activation to produce mediators, such that if in this period of heightened, physiologic responsiveness a secondary contact with a stimulant is made (not necessarily the same substance), an exaggerated inflammatory response is realized.

Once non-self molecules by either antigen specific or non-specific methods, it becomes necessary to translate that recognition into some means of biologic effector mechanisms and the unity in immunology is achieved by means of generating the very same type of effector molecules and processes regardless of the initiating substance.

11. Antigen-specific immunologists explain the differences in responses on the basis of past history of antigen exposure and types of immune response. For example, we are all exposed to ragweed pollen, but only a minority suffer harmful effects from it. Only some of us develop harmful IgE responses to the antigen. Those interested in occupational inhalational diseases are faced with a similar dilemma: out of all of the individuals exposed to occupational aerosols, how can we explain the fact that only some of those workers develop diseases such as hypersensitivity pneumonitis, toxic pneumonitis, or byssinosis?

The study of the biologic responsiveness to endotoxin affords us some possible answers to this question. Table IX lists a few of the ways that LPS can act as a trigger to hyper-stimulate cells or tissues already primed by another event. The examples in this table are selected and the list is by no means complete. Under this concept, certain unrelated events such as burns, abdominal surgery, unrelated infections, stimulation of other inflammatory mediators, etc. can prime certain responder cells and tissues in such a way as to render them physiologically altered. While temporarily in this altered state which does not last long, a "window of opportunity" exists such that if this individual is further stimulated, in our case, by endotoxin, heightened inflammatory are the result.

Inasmuch as such diverse phenomena as the Schwartzman reaction, endotoxin tolerance, adult respiratory distress syndrome, etc. all involve prior non-antigenic specific alteration by sensitizing factors, and all may involve endotoxin triggering, endotoxin inhalation biologists need to know what kinds of synergistic agents or events might prime an individual to be hyper-responsive to a coincidental endotoxin exposure. Obviously, no organic dust resulting from microbial decomposition or contamination is pure. Indeed, such dusts contain dozens of ingredients. We need to know more about synergistic responses induced by endotoxin and glucans or tannins, for example.

Stark and Jackson (reference 5) have formulated an attractive theory to explain why certain subjects hyperrespond to the effects of bacterial endotoxin. They state that conditions which favor the generation of oxidation products may sensitize mediator systems-. An increase in the poly-un-saturated membrane phospho-lipids composition enhances susceptibility because such fatty acids are easily oxidized to produce inflammatory mediators. Arachidonic acid metabolism and PAF synthesis are two important events arising from initial cell membrane perturbation. Interaction with key cytokines such as TNF and INF-G may ultimately be responsible for orchestrating these changes and thereby modify the host response to endotoxin. They further argue that any extrinsic factors that contribute to membrane lipid oxidation could render that individual hyperreactive to further insult.

12. Endotoxin biology also teaches us that endotoxin can prime inflammatory cells and tissues for hyper-responsiveness and many of these are listed in Table X along with the type of change in effects that have been measured.

From this table it is clear that endotoxin is extremely important in generating a wide variety of inflammatory responses of which the consequences might be severe. We need to know which of these kinds of events are capable of being produced in the lung following exposure to realistic amounts of aerosolized endotoxin. In these endeavors I extend those of you who are continuing the investigation much success in understanding this important substance.

References

Burrell R: Bacterial Endotoxin. IN: Comprehensive Toxicology, Sipes IG, McQueen CA, Gandolfi AJ, (Eds.). Oxford, Elsevier, P. In Press, 1997.

Burrell R: Human Responses to Bacterial Endotoxin. Circ. Shock, 43:137-153, 1994.

Burrell R: Immunomodulation by Bacterial Endotoxin. CRC Crit. Rev. Microbiol., 17:189-208, 1990.

Burrell R: Immunopharmacology of Bacterial Endotoxin. EOS - J. Immunol. Immunopharmacol., 11:85-90, 1991.

Stark JM, Jackson SK: Sensitivity to Endotoxin Is Induced by Increased Membrane Fatty-Acid Unsaturation and Oxidant Stress. J Med Microbiol, 32:217-221, 1990.

Table I. Inhalation-Related Diseases Associated with Airborne Endotoxin

Agriculture:
Swine/poultry confinement shelters
Poultry processing houses
Composting
Animal feed milling
Manufacturing:
Bioengineering - recombinant products
Machining oils
Mattresses making
Textile Production:
Cotton/spinning mills
Textile processing
Cotton garnetting
Carpet weaving
Flax processing
Miscellaneous:
Recreation - bath water
Offices serviced with humidification
Office buildings, mechanical ventilated equipment
Wastewater treatment

Table from reference 1

Table II. Summary of LPS-Binding Molecules

BINDING MOIETY	DESCRIPTION
Extracellular:	
High density lipoproteins	Initial detoxification
LPS-Binding protein (LBP)	Facilitates CD14 binding
Septins	Facilitate target cell binding
Cell Membrane Receptors:	
CD14	Binds to LPB-LPS complexes
73kDa glycoprotein	Binds LPS and peptido-glycans directly
CD11/18	Phagocytic ligand
Macrophage scavenger	Lipid A binder
KDO-binding receptor	
Lectins	Bind inner core regions

Described in reference 1

Table III. Site of Respiratory Tract Affected

Inhaled LPS Largely By-passes intravascular clearance mechanisms
Epithelial cells of conducting airways/alveoli
Endothelium of pulmonary capillaries
CD14 present on alveolar macrophages and on Types I and II epithelial cells
CD 14 ligand, LBP in BAL fluids
LPS-LBP complexes raise AM responsiveness 1000X thus reducing amount of LPS necessary to induce reactions. Increases stability of mRNA for TNF-α
Pulmonary surfactant proteins A (SP-A) and D
Enhances secretion of colony stimulating factor 7X
Inhibits SP-A enhanced phagocytosis of bacteria
SP-A molecularly similar to complement activator Clq
Clq receptor is the SP-A binding site on AM

Discussed in detail in reference 1

Table IV. Human IV Exposure Studies Use Standard Endotoxins

USA STUDIES:
Rudbach et al. 1976 prepared refined LPS from E. coli O113
Called EC-5 with a specific activity of 5 IU/ng
Deposited with the Division of Biologics of the US FDA
Almost all studies used EC-5 doses in 0.4-4.0 ng/kg range
Most use a single IV dose of 4 ng/kg or 20 IU
Highest dose tolerated by most volunteers
GERMAN STUDIES:
National reference standard LPS from Salmonella abortus-equi
Product electrodialyzed/ converted to a uniform sodium salt
Also use doses in 1-4 ng/kg range

Table V. Parameters of Human Intravenous Endotoxin Exposure

Fever, fever index
Blood Pressure
Complete peripheral blood count and differential
PMN responses to stimulants
CD2, CD3, CD4, CD8, CD14, CD20, HLA-DR
Secretion: IL-2, IL-1, TNF, IL-6, IL-8
Various prostaglandins, interferons, neurohormones
Complement
FEV_1 Pa_{O2}, Pa_{CO2}, clearance, IgG/alb ratios
Heart beat/pulse rate, vascular resistance index, etc
Markers of metabolic increase
Stress hormones
Gastrointestinal permeability
Coagulation/fibrinolytic products
Sleep parameters (REM/non-REM patterns)

Table VI. Endotoxin Aerosol Exposures, Human

Study	Agent	Findings
Paine	Serratia marcescens	↑ fever ↑ pulse ↑ resp. rate ↑ PMN ↑ Systolic B.P.
Cavagna et al.	"Purified E. coli ET"	↓ FEV1 > 6 hrs
Castellan Rylander	E. agglomerans LPS	↓ FEV1 ↓ CO D.C.
Rylander	E. coli	BAL: 100X PMN 3X lymphs ↑ AMϕ function ↑ Fibronection

For greater detail and discussion, see reference 1

Table VII. Caution for Laboratory Workers

Environmental ET easily contaminates reagents, glass, plasticware
Commercial reagents are often contaminated when obtained
Easy to inadvertently introduce ET subsequently, e.g.,: 5 ng/ml induces mononuclear cells to secrete leukocyte MIF But 10X concentrations inhibits the release
LPS monoclonal Ab or normal serum contaminated with ET were equally protective
Contaminated media - enhanced responses in G. pig AM0 cytokine production
1 pg/ml LPS induces cytotoxicity production by human monos
TNF-α assays frought with danger
Proteins from recombinant bacteria are often contaminated with ET
Skin test allergens often heavily contaminated with ET

Table VIII. Comparison of antigen-specific and antigen non-specific means of recognizing non-self

FEATURE	Ag-SPECIFIC	Ag NON-SPECIFIC
Stimulus:	Antigen	Immunomodulator
Ag Recognition:	Ig and TCR	Biochemical Receptors
Changes measured:	Efferent Responses	Afferent Responses
Secondary:	Anamnestic	Enhanced, non Ag-specific
Longevity:	Long,Ag-dependent	Temporary
Biologic Effect:	Cytokines, ToxOx Enzyme cascades Proteolytic enzymes Inflammatory cells	

Table IX. Lps Triggering of Pre-Primed Conditions

PRIMER	LPS TRIGGER EFFECT
Burns	Depress NK function ↑ Capillary pulmonary permeability ↑ Hypoxia, hypertension, thromboxane
Hyperoxia	↑ Mf-derived PMN chemotaxis ↑ PMN Alveolitis
IL-6	Synergistic TNF-α production
Γ - IFN	TNF-α (requires LAP) ↓ Cardiac output ↓ PO2 ↓ WBC count ↑ Capillary pulmonary permeability
Strep pyrogenic exotoxin	↑ febrile response
Muramyl di-peptide	Enhanced TNF-α
PAF	IL-1 production Arachidonic acid metabolism ↑ Thromboxane Superoxide production ↑ cAMP levels MPϕ activity TNF-α Leuko- and thrombocytopenia

Table X. LPS Priming

ENDOTOXIN PRIMES	EFFECTS
Macrophages	Primes PAF stimulation that leads to: ↑ Arachidonic acid metabolism Prostaglandins Leukotrienes
Human Monocytes	IFN-α only in presence of GM-CSF or IFN-Γ
Alveolar Macs	Adherence Peroxide, Superoxide anion PMN attraction IL-1 Leukotriene B4
Human PMNs	Enhanced respiratory burst Synergistic ↑ ToxOx metabolites Elastase-mediated endothelial injury
PMNs	↓ # /function of receptors: Leukotriene B4 C5a f-MLP ↑ Endothelial adherence (C11b/CD18)
PMNs	PAF generation
Atopic Basophils	Histamine release upon allergen stimulation
Astrocytes	Synergistic with Substance P IL-1 TNF-α
Sympathetic ganglia explants	Synergistic with IL-4 Substance P