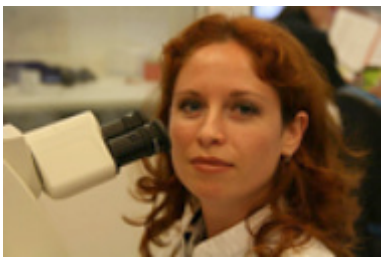
Bronchoalveolar lavage (BAL)



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Pulmonary diseases have traditionally been evaluated by laboratory tests, lung function tests, imaging procedures and tissue biopsies. Broncho alveolar lavage (BAL) represents an additional tool in the assessment of the health status of the lung for pulmonologists that can facilitate the diagnosis of various diffuse lung diseases. BAL is competent to provide cells and solutes from the lower respiratory tract. BAL fluid (BALF) can be analyzed to determine white blood cell (WBC) profiles and to detect respiratory pathogens. Although BAL is seldom useful as a “stand-alone” diagnostic test for the diagnosis of diffuse infiltrative lung disease, when combined with clinical data and high-resolution computed tomography of the chest, BAL WBC profiles can contribute significantly to the diagnosis of specific forms of interstitial lung disease (ILD). However, if despite this thorough clinical evaluation the diagnosis remains unclear, a biopsy should be considered as the final diagnostic step. Additionally, BAL can play a very important role in the diagnosis of respiratory infection, and it is useful in monitoring the lung allograft. Examination of BAL cells or acellular components of BAL via gene microarray technology or proteomic analyses may allow BAL to assume a more prominent role in diagnosis and management of lung disease in the near future. In the follow-up depicting prognosis and response to treatment BAL fluid analysis has less clinical relevance.

Clinical relevance of BAL

History of BAL as a diagnostic and research tool

Since the introduction of the rigid bronchoscope by Dr. Jackson in 1904, bronchoalveolar lavage (BAL) had become an increasingly important tool in pulmonary diseases. Primary, BAL was used as a treatment for patients who suffered from diseases associated with accumulation of purulent secretions such as alveolar proteinosis, cystic fibrosis and bacterial pneumonia. Large volumes of saline (15-30 L) were instilled over 2 to 3 hours to clear the lungs of excess secrete. This “large volume” BAL was commonly known as: bronchiolo alveolar debridement.
Between 1960 and 1970, the introduction of “small volume” (instillation of 300 ml sterile saline) BAL followed. Tested on healthy volunteers, it was found to be a save method, providing extensive information on cellular components of normal BAL fluid. After the introduction of the flexible scope in 1970, a increase in implementation and use of BAL was seen. The increase in interest in BAL as a research tool was reflected by the increase in publications (40-fold) on BAL in the period 1970-1990. In recent years, research output dealing with BAL has reached a plateau at approximately 500 papers annually.

Description of broncho alveolar lavage

By definition BAL is a method for the recovery of cellular and non-cellular components from the lower respiratory tract (e.g. alveoli). It is a safe technique, with few major complications. In many cases (e.g. pulmonary proteinosis, alveolar hemorrhage, eosinophilic pneumonia) BAL can replace lung biopsy. Possible uses of BAL in diagnostics are summarized in Table 1.

Table 1 shows pulmonary diseases where BAL fluid can be used to reach a diagnosis.

Non-infectious	Infectious
Sarcoidosis	(Ventilator-associated) pneumonia
Hypersensitivity pneumonitis	Pneumocystis pneumonia
Idiopatic longfibrosis	Mycobacterial infection
Connective tissue disorders	Aspergillus fumigatus infection
Langerhanscel histiocytosis	Viral pneumonia
Malignancies	Toxoplasma pneumonia
Alveolar haemorrhage	Legionella infection
Alveolar proteïnosis	Mycoplasma pneumoniae pneumonia
Eosinofilic pneumonia	Chlamydia pneumoniae pneumonia
Bronchitis obliterans with organizing pneumonia	Cryptococcal infection
Asbestosis	
Silicosis	

Technical aspects of broncho alveolar lavage

Guidelines and recommendations on the technical aspect of BAL have been published by the European Respiratory Society (ERS) Task Force in several reports. Premedication usually consists of a sedating compound (diazepam) with a compound that causes dilatation of the bronchi (atropine) and local anesthesia by application of lidocaine.

Site of lavage

The site of lavage depends on the localization of the abnormalities. In case of localized disease, for instance an infection with a radiographically apparent infiltrate or a malignancy, the involved segment should be sampled. In patients with diffuse lung disease, the middle lobe or lingula is most commonly used site to be lavaged since anatomically this is the most accessible site and the fluid obtained at one site is representative of the whole lung in diffuse lung diseases (inflammation is not limited to one site). Using the method described, approximately 1.5-3% of the lung (approximately 1,000,000 alveoli) are sampled.

Fluid used

Usually, the lavage is performed using sterile saline (0.9% NaCl). Preferable the saline is preheated to body-temperature (37°C) to help prevent coughing and to, slightly, increase cellular yield. However, this is not necessary and therefore many institutions use saline at room temperature.

The volume of sterile saline instilled differs between institutions, the volume varies between 100

and 300 ml in aliquots of 20 to 50 ml, the ERS task force recommended the use of 200-240 ml divided in four aliquots.

Fluid instillation and recovery

The fiberoptic bronchoscope is wedged into a subsegmental bronchus. The fluid is instilled through the bronchoscope and almost immediately recovered by applying suction (25-100 mmHg). Each aliquot is aspired into a separate syringe or trap. Recovered aliquots are consecutively numbered.
Usually 60-70% of the instilled volume is recovered in healthy volunteers, in smokers, patients with underlying pulmonary disease and ventilated patients, the recovery rate is lower.

Laboratory processing of BAL

BAL fluid is processed immediately upon arrival at the laboratory. Since the first aliquot is usually poorly recovered and reflects a disproportionate amount of bronchial material, this fraction is used it for mycobacterial investigation only. The other three fractions are pooled and processed. Complicated and time-consuming procedures, such as filtration, lysation and resuspending of BAL fluid can be avoided in order to sustain the cell morphology and to facilitate 24-hour service from the laboratory. Equal parts of fractions I, II and III are pooled and used for the further investigation, such as total cell count, cytospin preparations, quantitative culture and determination of soluble factors. When indicated additional tests can be included such as: detection of fungi, *Legionella pneumophila*, viruses and detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* by means of polymerase chain reaction (PCR).
The total cell count is performed by using a Fuchs-Rosenthal hemocytometer, every nucleated cell is counted. A BAL fluid sample retrieved from a healthy person contains 150.000 tot 300.000 cells per ml.

Quality control of BAL

To ensure that the obtained material represents the situation in the alveoli, a number of criteria have been established. A BAL fluid is regarded non-representative if it fulfills one of the following criteria: i) volume < 20 ml, ii) total cell count < 60.000 cells/ml, iii) presence of > 1% squamous epithelial cells, iv) presence of > 5% bronchial epithelial cells, v) presence of extensive amounts of debris, vi) severely damaged cell morphology.

Cytocentrifugation

All preparations are made by cytocentrifugation using a Thermo-Shandon Cytospin 3 (Thermo Electron's Anatomical Pathology Group, Astmoor, England) following a standardised protocol using pre-cleaned slides. The amount of BAL fluid (in drops) used is dependent on the number of cells per ml in the pooled fraction (table 2). The program used follows a centrifugation speed of 650 rpm (≈40 x g) with a low acceleration for 10 minutes. Using cytospin preparations shows a few advantages over smear preparations. First of all, the cytospin preparations are dry when they are taken out of the centrifuge, and can be fixed and stained immediately. Secondly, due to the monolayer, the preparation will stain equally and the contents of the cell and the nucleus are perfectly visible. The third advantage lies in the fact that a relatively small and easy-view preparation needs to be examined, making it easy to investigate the whole preparation.
However, there is a small disadvantage, BAL fluid differential cell counts on cytocentrifuged preparations can underestimate the proportion of lymphocytes.

Table 2. Cytocentrifugation parameters of the Cytospin 3 in the work-up of BAL fluid.

Cells/ml	**number of drops**	**Acceleration**	**Speed**	**Time**
< 50.000	7	Low	650 rpm	20 min
50.000 – 100.000	5 – 4	Low	650 rpm	10 min
10.000 – 200.000	4 – 3	Low	650 rpm	10 min
200.000 – 300.000	3	Low	650 rpm	10 min
300.000 – 400.000	3 – 2	Low	650 rpm	10 min
400.000 – 500.000	2	Low	650 rpm	10 min
>> 500.000	Dilute with NaCl 0.9% to 3 drops final volume	Low	650 rpm	10 min
BAL with excess amount of blood	use 1 drop less than mentioned above	Low	650 rpm	10 min

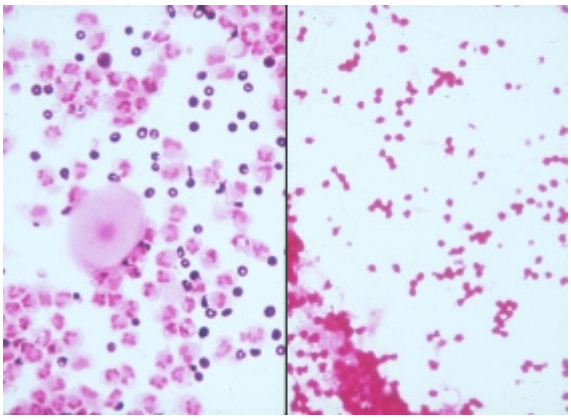

Figure 1. Gram stained preparations of a BAL fluid sample, showing that by using cytospin preparations (left) the nucleus and the cell contents are better visualized compared to smear preparations (right). Magnification 400x.

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disadvantage, BAL fluid differential cell counts on cytocentrifuged preparations can underestimate the proportion of lymphocytes.

Stains performed on BAL fluid

Many stains can be used in the work-up of BAL fluid. Careful analysis of the BAL fluid (BALF) cell profile and presence of a-cellular components in BALF can, combined with clinical and radiological features, help to ascertain a diagnosis. Application of BALF in the diagnosis of pulmonary infections has already proven to be very useful, specially in case of ventilator-associated pneumonia and opportunistic infections such as *Pneumocystis pneumonia*. A number of stains are routinely performed on each BAL fluid sample. These include the Gram-stain (one preparation) and the May-Grünwald Giemsa (MGG) (three preparations) stain. Before Gram-stain is performed there is a two minute fixation step with absolute methanol. Non-fixated preparations are used for the MGG stain.

After staining, each preparation is sealed by using a drop of Shandon-mount (Thermo Electron's Anatomical Pathology Group) and a covering slip. This ensures that the quality of the preparation does not deteriorate by the oil used for the oil-immersion lens, and that no damage occurs by used so that they can be stored indefinitely. Besides the standard stains, a number of stains can be added to the investigation upon clinical indication. For example: Grocott (methenamine-silver, fungi/Pneumocystis jiroveci), Auramine-Rhodamine, Ziehl-Neelsen (acid-fast bacteria), Legionella immuunfluorescence (Legionella spp), Acridine-orange (micro-organism in general) and the iron stain. Figure 2 shows a flowchart of the BAL fluid work-up as it is used in our laboratory in immunocomptetent intensive care patients on mechanical ventilation.

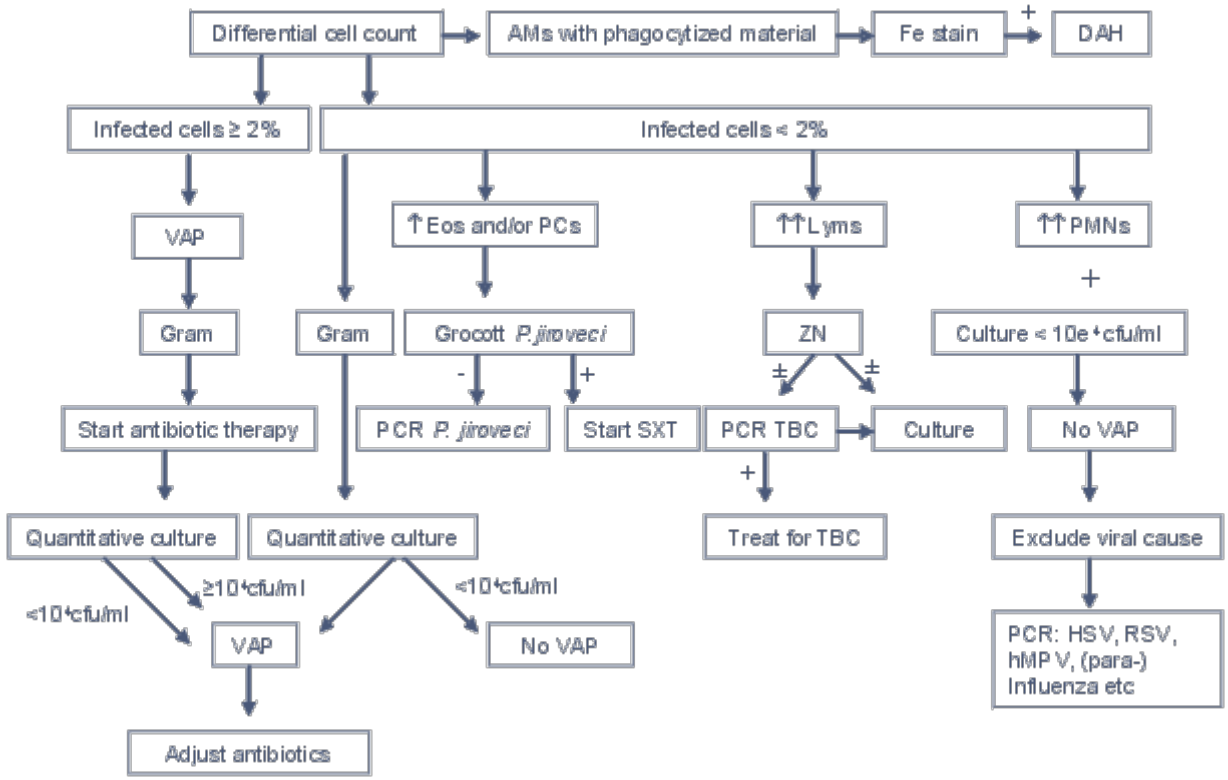
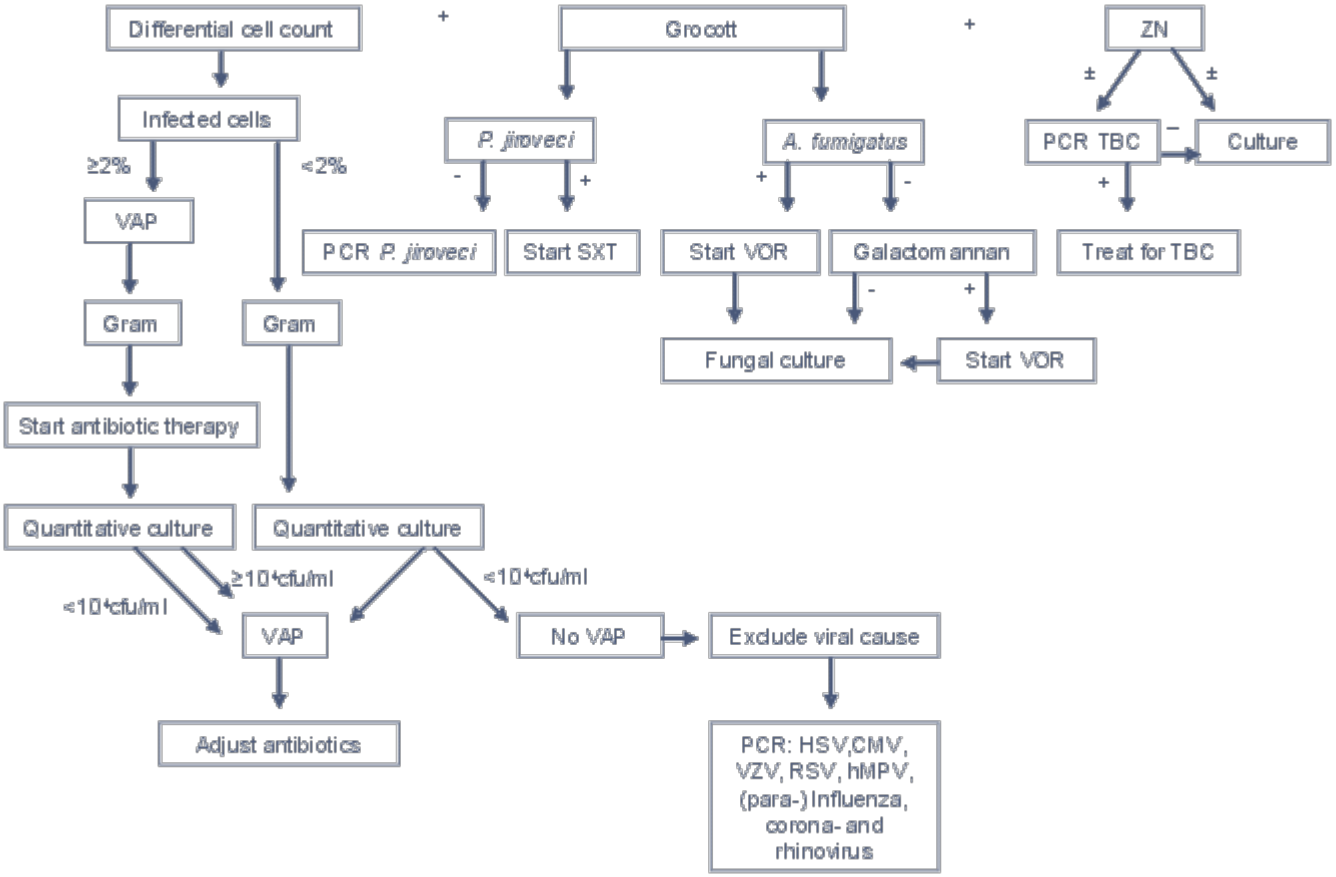


Figure 2. Flowchart showing the BAL fluid work-up in immunocompromised intensive care patients on mechanical ventilation.

Figure 3 shows a flowchart of the BAL fluid work-up as it is used in our laboratory in immunocompromised intensive care patients on mechanical ventilation.



The differential cell count

The differential cell count is performed on MGG stained preparations. When screening on low magnification (magnification: 100x), the presence of squamous epithelial cells, clusters of RPII cells, large clusters of *P. jiroveci* and mucus plugs can be noted. The differential cell count is performed using a magnification of 1000x and a standardized protocol. A total of 500 nucleated cells are counted, including the IC, and reported as a percentage of 500 cells. Specific morphological phenomena, such as foamy alveolar macrophages, activated lymphocytes, necrobiotic neutrophils and RPII cells, are reported separately. The differential cell count can be used as an instrument to limit the differential diagnosis in pulmonary diseases (table 3).

Table 3 shows the direction in which the elevation or the presence of certain cells in the differential cell count in BAL fluid can point.

	AMs	Lyms	PMNs	Eos	PCs	MCs	RPII
Non-infectious diseases							
Sarcoidosis		↑	=	=/↑	–	=/↑	–
Extrinsic allergic alveolitis	FAM	↑↑	↑	=/↑	+/-	↑↑	–
Drug-induced pneumonitis	FAM	↑↑	↑	↑	+/-	↑↑	–
Idiopathic pulmonary fibrosis		↑	↑/↑↑	↑	–	↑	+/-

BOOP*	FAM	↑	↑	↑	+/-	=/↑	
Eosinophilic pneumonia		↑	=	↑↑	+/-	=/↑	
Alveolar proteinosis	FAM	↑	=	=	–	=	
Diffuse alveolar haemorrhage		=/↑	↑	=/↑	–	=	+/-
ARDS**		↑	↑↑	↑	–	=/↑	+/-
Haematologic malignancies		↑	↑	=/↑	–	=/↑	
Astma		=	=	↑	–	=	
Infectious diseases							
Ventilator associated pneumonia		=	↑↑	=	–	=	+/-
Pneumocystis jiroveci pneumonia		=/↑	↑	=/↑	+/-	=	+/-
Viral pneumonia		=	↑↑	=	–	=	+/-
Aspiration pneumonia	FAM	=	↑↑	=	–	=	+/-

AMs: alveolar macrophages, Lyms: lymphocytes, PMNs: polymorphonuclear neutrophils, Eos: eosinophils, PCs: plasma cells, MCs: mast cells, RPII: reactive type II pneumocytes, FAM: foamy alveolar macrophages, +: present, -: not present, +/-: can be present; * BOOP: bronchiolitis obliterans with organising pneumonia; ** Adult respiratory distress syndrome.

The quantitative culture

The quantitative culture of BAL fluid is the current “gold standard” for the diagnosis VAP. Since the alveoli are rinsed with approximately 200 ml of fluid, the actual BAL fluid sample is an approximately 10-100 times diluted representation of the situation in the alveoli. In case of a bacterial infection, the bacterial concentration in the alveoli is 105 tot 106 colony forming units (cfu)/ml. For the quantitative culture, this results in a cut-of value of 104 CFU/ml.

Molecular techniques in BAL fluid work-up

In recent years, the introduction of molecular methods in the diagnostic work-up of respiratory infectious diseases has increased. Molecular techniques, such as polymerase chain reaction (PCR) offer some advantages over conventional techniques (culture, serology). First, the use of PCR leads to an increase in sensitivity, especially in micro-organisms that cannot (easily) be cultured and in case of a low burden of micro-organisms. Furthermore, it is a universal technique making it possible to identify every micro-organism, using unique primers and probes, making it very specific. Finally, PCR is a relatively rapid method for identification compared to culture and serology.

Several PCR methods can be useful in the identification of causative organisms in pulmonary infectious diseases. In case of a conventional PCR method, post-PCR products are analysed by means of gel-electrophoresis, enzyme immunoassay detection or dot-blot hybridisation. In recent years, the conventional PCR methods are increasingly replaced by real-time PCR reactions which do not require separate post-PCR product analysis. This has led to results being

available earlier and with less chance of contamination since there is no need to open PCR tubes after amplification. An additional advantage of real-time PCR methods is the fact that this method measures the amount of amplified PCR product during each cycli. This makes it possible to obtain quantitative results, which is essential when identifying micro-organisms which can lead to either carrier state or infection (e.g. *P. jiroveci*).
Combining diagnostic PCRs to identify different micro-organisms at the same time may be the future since respiratory pathogens can cause the same clinical symptoms. Using PCR assays which only detect one micro-organism may prove to be too expensive and require too much material. Multiplex PCR reactions permit the amplification and identification of multiple pathogens simultaneously, however in general, they are less sensitive compared to mono-specific PCRs. PCR can be used for the identification of many causative organisms of pneumonia. Table 6 sums the most prevalent, causative micro-organisms of pulmonary infection for which a PCRs is described in literature.

Table 6 PCR assays described in literature for different micro-organisms causing pulmonary infection.

Micro-organisms
Bacteria:
Streptococcus pneumoniae
Haemophilus influenzae
Mycoplasma pneumoniae
Chlamydia pneumoniae
Legionella pneumophila
Fungi :
Pneumocystis jiroveci
Aspergillus fumigatus
Viruses :
HSV-1,2
RSV
HMPV
Influenza A,B
Parainfluenza 1,2,3

* for detection in respiratory samples

Bronchoalveolar lavage

By Herbert Y. Reynolds.

Bronchial lavage of the human airways, as therapy to remove secretions, dates to the early use of the rigid bronchoscope over a century ago. And for the past 50 years, lung biological specimens have been obtained also for research by washing airways through rubber tubes anchored in the bronchi. Limited lavage in normal subjects to recover airway samples for research began in 1967. Availability of the flexible bronchofiberscope in the late 1960's popularized the use of bronchoscopy, and the recovery of lung washings for clinical analysis and research studies was done frequently. We studied airway cells and protein and immune components in lavage fluid from normal subjects to describe the lower respiratory tract milieu in 1974. Subsequently, the use of bronchoalveolar lavage (BAL) to retrieve specimens from patients with many forms of lung disease and from controls or normals spread quickly throughout the US, Europe and Japan. There was sufficient interest in presenting and discussing lung research prompted by BAL that the first BAL Conference was held in 1979. Dr. Ronald Crystal, Dr. Anthony Kalica and I organized the second BAL Conference in Columbia Maryland in 1984. Nine others have followed including the 11th BAL Conference in Athens, Greece on June 19-21, 2008, which is the subject of this report.

11th BAL International Conference

This was the first joint BAL and WASOG meeting, and this was a plausible plan as there was considerable cross over between the research findings that used BAL sampling to obtain biological specimens from the lung and others presented that analyzed and interpreted lung-derived biomarkers found in many interstitial lung diseases (ILD). Thus, the respective BAL and WASOG presentations interdigitated well. Among the formal presentations at plenary sessions, 18 were related to BAL sampling. Among almost 100 posters, 25 were research studies involved with BAL. The posters were all presented by their authors during several organized "poster rounds"; three were selected for oral poster presentations in plenary sessions. This analysis will emphasize the oral BAL studies.

In organizing a summary of noteworthy contributions from BAL studies, these have been grouped into 6 categories:

1. **Analysis of BAL findings to evaluate disease(s)**
   The study of cells and proteinacious substances in lung washings continues to provide insight into the pathogenesis and host immunity responses involved in inflammation, fibrosis, acute injury, asthma, and infection. Because components retrieved in BAL fluid are in close proximity to diseased tissue and are the first approximation to in situ occurring events, relevant new observations were given. Dr. V. Potelli (Italy), discussed BAL findings in acute lung injury that might be a surrogate for a lung tissue biopsy. Dr. D. Israel-Biet (France), evaluating patients after lung transplantation for graft rejection and

onset of the bronchiolitis obliterans syndrome, looked for markers of these conditions; persistence of polymorphonuclear neutrophils appeared to be a signal. As environmental exposure is a common cause or confounding factor in establishing a precise etiology of ILD, the BAL findings were presented for several common diseases. Dr. S. Constantopoulis (Greece) assessed the role of BAL in non-occupational asbestos exposure, Dr. F. Kokknis presented about chrysotile exposure, and D. F. Evyapan did so for metal induced lung disease. Dr. M. Drent (Netherlands) updated the usefulness of a computer data bank of BAL components available for analysis in establishing a diagnosis of a particular ILD.

2. **Technology applied to BAL continues to evolve**
   New analytical modalities continue to reveal more biomarkers and mechanisms defining cellular activities. With the expanding "omics" approach to biologic materials a much more comprehensive analysis of cellular output and gene displays in BAL fluid and cells can be viewed from a normal or patient specimen. Dr. P. Rottoli, (Italy), a pioneer in proteomics, continues to use this tool to search for biomarkers in ILD. Similarly, gene arrays from cells, as describe by Dr. N. Kaminski, (USA), sampled from lung tissue, BAL, and blood described cellular activity in ILD and for many other diseases.
3. **Timing of BAL fluid samples may reveal an evolution of changes**
   As BAL is well tolerated by normals (controls) and patients, the samplings can be repeated. This may have value in monitoring changes in BAL components during early lung development and subsequent aging, and during persistent illness. Dr. D. Phelps (USA), describe age-related changes in BAL components with a rat model. Similarly, Dr. N. Kaminski (USA) discussed serial analyses on blood cells, but which could apply to BAL cells. Longitudinal evaluation to monitor airway BAL changes is to be encouraged in patients with evolving or persistent illness, and in volunteer controls to observe changes that occur normally with aging. Providing BAL data from older control subjects would better approximate or contrast with these found in older patients. Controls for research often need to be better age-approximated with patients, particularly in metabolomic comparisons and in drug turnover rates when pharmacomics are investigated.
4. **Special cells isolated from BAL**
   Lung lavage retrieves important detachable types of cells and other biological specimens for in vitro study, especially alveolar macrophages (8) and lymphocytes. Thus, a method to isolate dendritic cells (DC's) from BAL fluids was of particular note (9). Dr. T. Berge and colleagues (Netherlands) and Dr. B. Lambrecht (Belgium) presented a method to obtain dendritic cells; they estimate that about 0.1% of normal lavage cells are DC's. The ability to recover DC's among alveolar cells, which are mostly macrophages, provides a means for dissecting the innate immunity processing that begins in the alveolar space. The in vitro study of alveolar macrophages as phagocytes and effector cells in initiating inflammation and alveolitis has been considerable. But, Dr. C. Saltini's (Italy) use of them to reproduce the kinetics of tuberculosis infection, seemingly replicates an "in vivo" approach to creating a Mycobacterium exposure to AM's with subsequent infection of 40% of these cells and then elucidating their production of inflammatory cytokines. This seems the way tuberculosis may begin in a susceptible person.

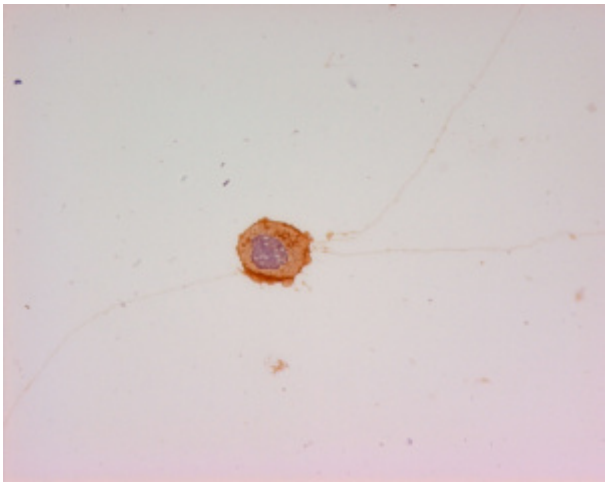


Fig. 1 A 400x magnification of a dendritic cell isolated from human bronchoalveolar lavage, stained with DAP anti-HLA-DR showing the long typical dendrites.

5. **Perturbations in animals or humans that affect BAL components**
   Inhalation of cigarette smoke, exposure to various environmental toxins, often occupation-related, and aspiration of refluxed gastric secretions, all induce special changes in the composition of BAL components. Another exposure illustration with ozone by Dr. J. Floros (USA) created oxidative stress in the airways that was found to effect surfactant protein A. The result was illustrated with a proteomics analysis that introduced the term “discovery proteomics.”
6. **Use of BAL in children**
   As this procedure is finding more applications in young patients, now performed shortly after birth in some with congenital diseases such as cystic fibrosis, and in children with asthma or interstitial lung diseases, the safety and consequences of the lavage procedure require continued surveillance. It was in part this consideration of safety with research use of investigative fiber -optic bronchoscopy, often coupled with broncho-provocation to simulate asthma and then performing lavage, and/or other procedures done such as airway brushing and transmucosal or transbronchial biopsies that prompted the National Heart, Lung, and Blood Institute and the National Institute of Allergy and Infectious Diseases to convene a workshop to review the scientific merit gained from this approach. An Appendix is available online in the journal issue (www.atsjournals.org) that was included with the Workshop Summary (titled: Appendix II: “Ethical Issues Related to Bronchoprovocation and Bronchoscopy Research”). The consensus among the workshop participants was positive that the risks compared with new scientific information obtained was worthwhile; continued use of this form of research was reaffirmed. But, there were some concerns about adequate training of personnel performing these procedures, and obtaining surrogate permission for these young, vulnerable patient subjects that should be reviewed for possible updating. Other issues considered included: informed consent, financial incentives, and additional protections for research involving children and those subjects who need surrogate permissions.

What might be future expectations from BAL analysis?
In several years, a next BAL Conference will occur. What should we expect this sampling procedure to yield?

1. **Continued revelations from research on BAL samples will continue**
   As this method of obtaining airway-alveolar space biological specimens already has contributed many important insights into the normal and diseased respiratory tract, more scientific observations will be forthcoming. Although this sampling method is termed "BAL," operationally the procedure is broader and encompasses other strategies which should be given more innovation, such as local sampling in large versus peripheral airways, analysis of exhaled breath condensates, comparisons between blood and airway cells and proteins, and genetic arrays of respiratory cells. As the "omics" wave of measurement technologies continues, the discovery of new substances or cellular functions will enhance the understanding of many diseases. Perhaps more research will occur that compares the upper respiratory tract secretions and cells with the lower tract that might find similarities that would promote more upper airway sampling. This could facilitate less invasive longitudinal monitoring, be acceptable for children and other younger subjects, and explore diseases that affect both portions of the respiratory tract, such as allergic rhinitis-sinusitis and hyperactive airways of asthma syndromes.
2. **Training of future respiratory clinical scientists who use respiratory tract sampling methods**
   The continued output of relevant scientific research on respiratory tract diseases is predicated on having a sufficient supply of interested, well trained, innovative investigators. How we motivate and support this next generation is an important task for the academic research community. The success to fund and train these investigators is a collective effort between the supporting agencies, the professional societies and specialty groups, and the pharmaceutical industry. An assessment of "how are we doing" might be considered. The BAL Conference with it's broadly representative and international composition of participants might consider a formal review of some of these training topics.
3. **BAL will help to reveal "secrets" still in the lungs**
   As it remains surprising what "we don't know" about cells in the respiratory tract and their functions, a NHLBI workshop was held (July 9-10, 2007) to explore how more information can be obtained about still unrecognized and insufficiently studied cells. The summary of this workshop and companion papers that review in detail the development of the lung, the airways, the alveolar unit, and the pulmonary vasculature will each present research recommendations. The use of BAL and other local sampling methods to retrieve cells and other biological specimens will be needed. Future BAL conferences will likely deal with these new findings, as the lung's cellular secrets are revealed.