CHAPTER 7

IN VIVO STUDIES

7.1 INTRODUCTION

Many approaches involving the drug inhalation aerosols have been attempted for the treatment of asthma, chronic bronchitis, emphysema, cystic fibrosis, pulmonary infections and other diseases (Smith et al, 1996). This is because of possibility of significantly reducing the clinically effective dose and, thus improving drug safety via topical administration to the lung (Gupta et al, 1997). Inhalation is essentially a method for topical application of drug to the airways; the surfaces on which the drug is deposited are inaccessible to direct observation. But the effective distribution of drugs to their sites of action in the lungs and nasal passages remains an important parameter in the development of inhaled pharmaceuticals. To achieve clinical success, distribution is probably second in importance only to the pharmacology of the drug that is administered.

To determine the amount of drug deposited on an internal surface and its relationship to the therapeutic effects is not an easy task. Traditionally, pharmacokinetic measurements are used in which the amount of drug in plasma is analyzed as a function of time. This is an indirect measurement which requires a series of experiments to separate the contributions from deposition of drug on the target tissues (lung,sinuses, turbinates) and non-target tissues (mouth, GI tract). Then, traditional pharmacokinetics assume that the relative concentration of drug in different tissues is fairly constant. Those relationships are constant for drugs that are injected, but may not be at all constant for inhaled drugs. Deposition of an inhaled drug is subjected to intersubject and intrasubject variability and plasma pharmacokinetics may not be a reliable indicator of deposition. Inhaled drug which is observed in the plasma may or may not have been in the lung at a previous time. When drug is observed in the plasma it is no longer very therapeutically effective. The plasma curve contains information relevant to safety, but it can only address efficacy by a complicated chain of inferences except for drugs and therapeutics administered by inhalation for systemic application (e.g Insulin). Therefore, deposition is an important parameter for any inhaled drug, and is of critical importance for drugs such as antibiotics and anti-infectives meant for local action. Estimation of regional distribution of inhaled antibiotics would give the true picture of deposition pattern at target site. In vivo regional distribution, pulmonary clearance, alveolar macrophage uptake and pulmonary toxicity of TB/AMK-ALLP were evaluated in rat and compared with their respective lactose base formulations.

7.2 METHODS

White albino rats of either sex (weight 190-250 gm) were used for the study; animals were housed in controlled environmental conditions with normal hygiene and normal diet at a 12 hr light/dark cycle and were provided water *ad libitum*. Experimental protocol was approved by the institutional ethical committee of The Maharaja Sayajirao University of Baroda, Vadodara, India. Animals were divided into two groups. Animals were divided into two groups of 3 animals each to receive different treatments. Group 1 administered the TB/AMK ALL and group 2 administered the conventional lactose base formulations.

For comparison purposes, conventional lactose base formulations (LBF) of TB and AMK were prepared by spray drying aqueous solution of TB/AMK and lactose together. Drug proportions on above formulations were at the same level with their corresponding ALLP formulations selected for in-vivo study. These particles possesses mean geometric diameter of 3.14±1.2 μm, 4.26±1.12 μm, bulk tapped density of 0.47± 0.03g/cm^3 ,0.51± 0.04g/cm^3 and fine particle fraction (FPF) of 27±1.26 % and 26.4±2.1 % for TB and AMK- LBFs.

7.2.1 Intratracheal Powder Administration

Either ALLP or LBF were administered through an exposed trachea of anaesthetized animals. Animals were anaesthetized using intra peritoneal injection of 1.2 gm/ kg of urethane. A small rigid tube (approx 1 mm outer diameter) was loosely inserted between two cartilaginous rings to the lumen of exposed trachea above the carina. This tube was then connected to an Ugobasile rodent ventilator via inhalation and exhalation ports. 20 mg of either ALLP or LBF were placed in the inhalation port and insufflated into the lungs of animals by forced ventilation at a 3 ml tidal volume with 75 strokes per minute. Following the short period of pulmonary delivery the tube was removed from the trachea with no sign of injury.

7.2.2 Assessment of Pulmonary Deposition

For assessment of regional lung deposition, the lung was divided into trachea (with main bronchi) and pulmonary lobes. Each lobe was cut in two equal parts by mass: one central part cut round in shape around the bronchus hilum, and one peripheral part; and the different tissue samples were finely minced and drug content was estimated. To compare the efficiency of deposition of the different formulations in the lung, several parameters were measured. (1) The deposition in the trachea (T), central (CS), and peripheral (PS) lobe sections was expressed as percentages of total deposition within the lungs (Neer et al, 2001). Because the proportion of lung parenchyma (the preferential site of absorption to the systemic circulation) to conducting airways is larger in the peripheral lobe section compared

to the central lobe region, the ratio of deposition in the peripheral to central lung (which included the central lobe section and the trachea; P/C ratio) was used as an index of deep lung deposition and was calculated as [PS/ (CS+T)]. A "P/C ratio" close to 1.0 indicates a homogeneous deposition within the lung lobes and limited deposition in the trachea, whereas a ratio close to 0.0 indicates a preferential deposition in the trachea and the central lobe section (Codrons et al, 2004).

7.2.3 Pulmonary Clearance Study

Animals were divided into two groups (Group1&2) and each group further sub- divided into 7 sets of 3 animals each. Group 1 administered the 20 mg of TB/AMK-ALLP and group 2 administered the same quantity of conventional LBF. Designated set of animals were sacrificed at predetermined time interval of 0, 1 ,2,4, 8, 12 and 24 h. Whole lung was removed, finely minced and ground for 2 min in 10 ml of PBS with a tissue homogenizer and drug content was estimated by HPLC.

7.2.4 Alveolar Macrophage Uptake Study

The lung of rats at end of 1 hr of powder administration was lavaged three times with 5.0 ml of phosphate-buffered saline (PBS) maintained at 37°C. The resulting fluid was centrifuged at 5000 rpm for 10 min in a refrigerated (4°C) centrifuge to separate the cells from supernatant containing various surfactant and enzymes. The cell pellet was separated and washed twice with PBS in cold condition and observed under phase contrast microscope to observe particle uptake by the alveolar macrophages.

7.2.5 Assessment of Pulmonary Toxicity

Pulmonary toxicity was assessed by analysis of cellular and fluid components of bronchoalveolar lavage fluid (BALF). Assisted ventilation (sham operated) animals were used as non treated control. The airways and lungs were washed three times with 5.0 ml of PBS until a total volume of 15 ml. The recovered BALF centrifuged and the supernatant removed. The parameters examined were total proteins, indicative of transudation of serum proteins across the capillary barrier, lactose dehydrogenase (LDH) indicative of general cell injury and acid phosphatase (AP), a lysosomal enzyme which is released during the phagocytosis and/or macrophage and neutrophil damages. If increased level of these enzymes detected, indicates cell injury. The palletized cells were re-suspended in PBS and counted by hemocytometer. Cytocentrifuge preparations were stained with Leishmann's stain for differentiation of white blood cell types. Examination cell population such as total cell, macrophages, neutrophils and eosinophils is a good indicator of potential pulmonary damage.

Biochemical assay in BALF, such as LDH and AP activities were assayed by the methods of Wotton (1964) and Moss (1984) respectively.

Histopathology of lung was carried out after 24 hrs of ALLP administration. Animals were sacrificed; respiratory tract tissues were immediately excised. Trachea were removed and fixed in 10% neutral buffered formalin (NBF). The lungs were gently inflated with 10% NBF fixative for 48 h. Tissues were processed, sectioned and stained with heamatoxylin and eosin. Stained sections were examined for evidence of local toxicity (minor pathological change) by an independent pathologist using light microscopy.

7.2.6 Data and Statistical Analysis

For drug targeting or drug delivery in general it is important to be able to quantitatively assess the site-targeting effectiveness (Bodor et al, 2003). Each testing was carried out three times and data from all experiments are expressed as mean ± SD unless specified. The statistical analysis of the data was carried out using ANOVA and unpaired student's t-test. $P<0.05$ were considered to be significant.

7.3 RESULTS AND DISCUSSION

To understand the site of deposition of ALLP, the regional distribution was assessed and compared with conventional lactose base formulations with in the lungs. The percentages of tracheal, central, and peripheral deposition relative to total recovery of the ALLP and LBF are recorded in Table 7.1 & 7.2. Significant difference ($P<0.05$) was observed in the deposition pattern of ALLP and LBFs. For both TB and AMK-ALLP, almost 53-58% was found in the right and left lungs against 22-26% with LBFs. The percentage of central deposition relative to total deposition reached 31.70±1.26%, 31.75±2.27%, 16.31±1.18 % and 14.05 ±1.58 % for TB-ALLP, AMK-ALLP, TB-LBF and AMK- LBF respectively. The corresponding P/C ratios were 0.43±0.06, 0.44±0.05, 0.17±0.05 and 0.15±0.04 respectively. TB and AMK-ALLP shown superior aerosol performance and more homogenous deposition with in the lung with respect to their LBF. As evident from Figure 7.1, central lobe region has high deposition fraction as compared to peripheral region. Central lobe region is rich in conducting airways (Patton et al, 1994) and is the target site for treatment of diseases involving infection of the airway (cystic fibrosis, bronchiectasis) without substantial systemic component (Vanbever et al, 1999). High deposition fraction in the central lobe region may reduce the therapeutic dose of antibiotics and anti-infectives intended for local action.

The high fine particle fractions of the ALLP measured *in vitro* translated into significant respirable fractions *in vivo* in the rat. The fraction of particles with an aerodynamic size <5

μm was 62–64% in the eight-stage cascade impactor using a Rotahaler device at an airflow rate of 28.3 L/min. The total fraction of the delivered ALLP mass that was recovered from the lung lobes reached a relatively close value of 53-58%. It is noteworthy that dry powder dispersion and penetration in the lungs were not dramatically impeded by the high relative humidity of the respiratory tract animals (Menache et al, 1995).

Table 7.1 Deposition pattern of TB-ALLP and TB-LBF in rat lung (Mean±S.D, n=3)

Tissue	*TB-ALLP*				*TB-LBF*			
	Distri-bution (%)	**C (%)**	**P (%)**	**P/C ratio**	**Distri-bution (%)**	**C (%)**	**P (%)**	**P/C ratio**
Trachea and main bronchi	44.62 ±5.16	-	-		74.65 ±6.20	-	-	
Right lung								
Anterior lobe	4.75 ±1.46	2.74 ±0.56	2.0 ±0.87	0.43 ±0.06	2.86 ±1.43	1.72 ±0.68	1.26 ±0.28	0.17 ±0.05
Middle lobe	6.91 ±2.57	3.80 ±1.67	2.91 ±0.88		1.20 ±0.32	1.08 ±0.14	0.46 ±0.33	
Posterior lobe	17.25 ±3.16	11.56 ±2.28	5.72 ±1.11		8.24 ±2.81	5.52 ±1.44	2.78 ±1.65	
Accessory lobe	12.38 ±2.59	6.26 ±1.74	4.7 ±1.22		6.47 ±1.46	4.12 ±1.10	2.98 ±1.24	
Left lung	14.09 ±4.73	7.34 ±2.52	6.62 ±2.45		6.58 ±1.57	3.87 ±1.70	2.65 ±0.66	
Total	100	31.70 ±1.26	22.69 ±2.70		100	16.31 ±1.18	10.12 ±2.08	

C-Central lobe deposition, **P**-Peripheral lobe deposition

Table 7.2 Deposition pattern of AMK-ALLP and AMK-LBF in rat lung (Mean±S.D, n=3)

Tissue	*AMK-ALLP*				*AMK-LBF*			
	Distri-bution (%)	**C (%)**	**P (%)**	**P/C ratio**	**Distri-bution (%)**	**C (%)**	**P (%)**	**P/C ratio**
Trachea and main bronchi	46.74 ±4.81	-	-		77.94 ±7.26	-	-	
Right lung								
Anterior lobe	5.24 ±0.66	3.16 ±0.87	2.43 ±1.02	0.44 ±0.05	2.23 ±0.32	1.16 ±0.72	1.12 ±0.71	0.15 ±0.04
Middle lobe	3.64 ±1.59	1.97 ±0.56	1.70 ±0.57		1.07 ±0.24	0.79 ±1.57	0.34 ±0.42	
Posterior lobe	14.51 ±3.75	9.37 ±2.14	5.67 ±2.14		7.24 ±1.06	4.85 ±3.14	2.53 ±1.31	
Accessory lobe	12.22 ±1.96	6.84 ±1.42	5.46 ±1.32		5.22 ±1.42	2.97 ±1.27	2.61 ±1.30	
Left lung	17.65 ±2.34	10.41 ±2.74	7.24 ±2.68		6.3 ±1.16	4.28 ±2.06	2.12 ±0.81	
Total	100	31.75 ±2.27	22.50 ±1.64		100	14.05 ±1.58	8.72 ±1.75	

C-Central lobe deposition, **P**-Peripheral lobe deposition

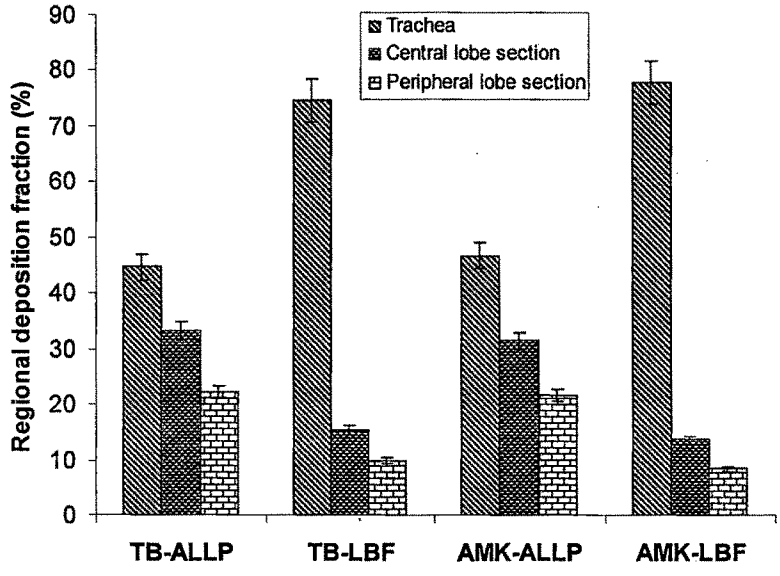


Figure 7.1 Regional deposition of TB/AMK formulations in the rat lung (Mean±S.D, n=3)

The concentration of TB/AMK in lung tissue was determined following administration of ALLP and LBF of TB/AMK. The total TB/AMK concentrations in the lung are collected in Table 7.3 and plotted in Figure 7.2&7.3. The lung concentration of TB/AMK remains high for long period of time with ALLP formulations. Over the 4 h following administrations of LBFs, the drug load in the lung decreased to about 23% of the initial drug load. After 4 h, the levels of TB/AMK in the lung tissue were below the limit of quantification. In contrast when TB/AMK was administered as ALLP, the level of TB/AMK remains well above the quantification level for at least 12 h of post administration, indicating reduced pulmonary clearance of drug from ALLP. It is possible that the maintaining of high level of antibiotic concentration over sustained period may be partly due to the more slowly cleared of ALLP, because of the role of large particles in escaping phagocytosis (Edwards et al, 1997 & 1998). This suggests that the delivery approach may require infrequent dosing to achieve the desired clinical effect. In addition, this retained pulmonary antibiotic concentration may also reduce the potential buildup of resistance due to the reduced need for patient compliance in maintaining high levels of drug over time. This profile of increased lung antibiotic concentration over a sustained period of time could be advantageous in reduction or prevention of selective bacterial resistance when using aminoglycosides, as this type of antibiotic exhibits concentration-dependent killing with time (Vogelman et al, 1986 and Schentag et al, 1997). In fact, dosing regimens of targeting high peak concentration relative to MIC appear to yield the best clinical outcome (Karlowsky et al, 1997).

Table 7.3 Total rat lung drug concentration over time (Mean± S.D, n=3)

	mg/total lung			
Time(hr)	**TB-ALLP**	**TB-LBF**	**AMK-ALLP**	**AMK-LBF**
0	8.04±4.16	7.94±4.42	8.41±3.84	8.17±4.06
1	7.81±4.27	7.23±3.59	8.07±4.49	7.63±3.66
2	5.97±2.8	5.16±2.64	7.29±3.17	6.16±3.28
4	3.87±2.07	1.84±1.22	4.26±2.25	1.91±1.10
8	3.15±1.29	0.71±0.46	2.58±1.29	1.15±0.74
12	2.1±1.25	0.67±0.16	2.44±1.22	0.79±0.64
24	1.26±0.48	0.65±0.12	1.48±0.67	0.58±0.43

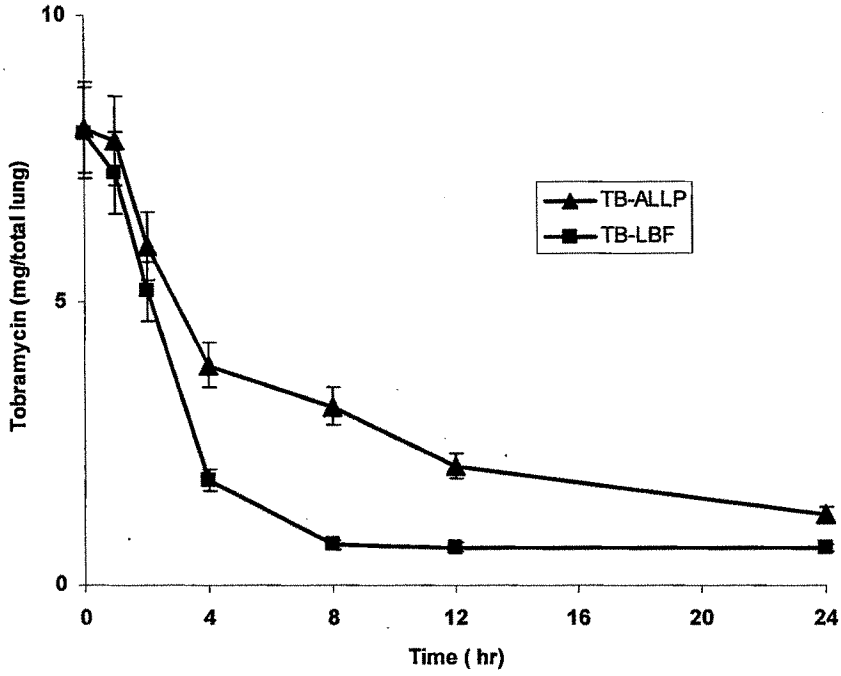


Figure 7.2 Total rat lung Tobramycin concentration Vs Time (Mean± S.D, n=3).

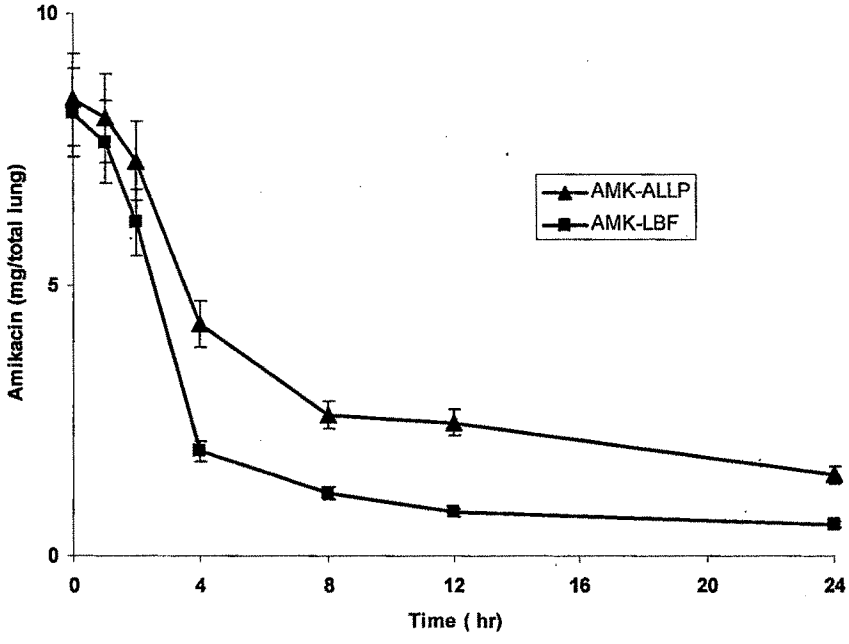


Figure 7.3 Total rat lung Amikacin concentration Vs Time (Mean± S.D, n=3).

Interaction of alveolar macrophages and ALLP was investigated *in vivo* and was compared with conventional LBFs. Particle uptake was studied under phase contrast light microscopy. Figure 7.5 & 7.6 shows photomicrographs of rat alveolar macrophages exposed to conventional LBF and ALLP. After 1h of administration 17.82 ±4.3% of phagocytic cells contained TB-LBF and 21.6 ±3.2% contained AMK-LBF. By contrast, only 11.5 ±3.4% of phagocytic cells contained TB-ALLP and 9.63 ±3.5% contained AMK-ALLP. For LBFs, 19.77±2.5 % (TB-LBF) and 24.8±3.7% (AMK-LBF) of the phagocytic cell population contained three or more particles 1h after inhalation, compared with 5.86± 1.8% (TB-ALLP) and 4.62±1.3% (AMK-ALLP) for large particles. These results are consistent with earlier findings that phagocytosis of particles diminishes precipitously as particle diameter increases beyond 3 μm (Kawagichi et al, 1986).

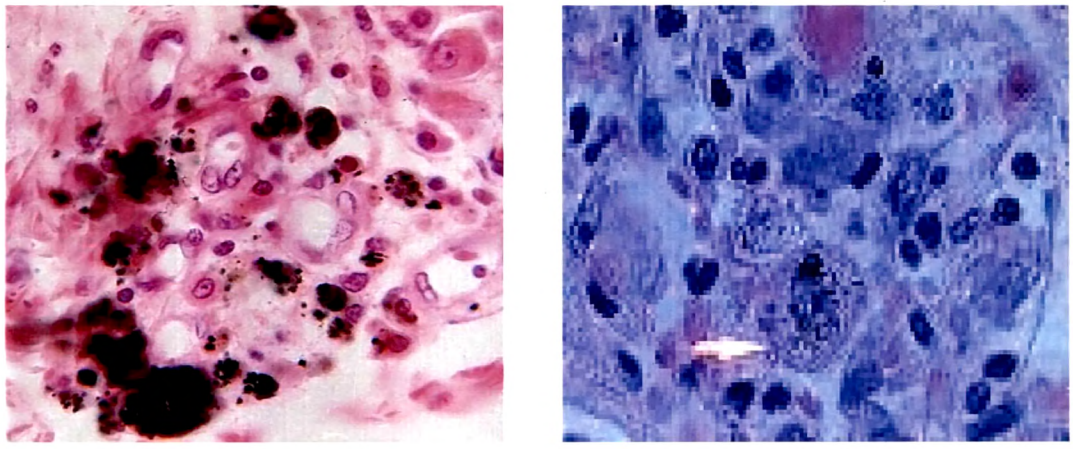
LBFs showed evidence of a numerous particles within the cell cytoplasm (Figure 7.5A & 7.5B), whereas reduced phagocytic uptake (0-2 particles per cell) was observed with ALLP (Figure 7.6A & 7.6B). Maintaining of high level of antibiotic concentration over sustained period is desired for the reduction or prevention of selective bacterial resistance, it must be retained in the lung for a prolonged period .When foreign particulate material reaches the alveoli, where no ciliated epithelium is present, deposited particles may stay for longer times. However, in the alveolar region of the lung, microparticles come into contact with another lung defense mechanism, the alveolar macrophages. Therefore, to enable such prolonged lung antibiotic concentration requires the ability of particles to escape detection and up-take by alveolar macrophages.

Changes in cell populations in BALF, AP and LDH levels, following particle inhalation are recorded in table shown in Table 7.4. As indicated in Figure 7.7 there was no significant difference ($P>0.05$) in the cell recovery, whether macrophages, neutrophils or eosinophils. This was similar with sham operated animals, and those who inhaled TB/AMK LBFs, TB/AMK-ALLP. Similarly, the data indicate that no significant difference in total protein (about 23 mg for sham operated, 21-26 mg for treated animals), AP (about 16 units for sham operated, 14-16 units for treated animals) and LDH (about 102 units for sham operated, 97-100 units for treated animals) levels. These suggests that inhalation of either LBF or ALLP did not produce either pulmonary inflammation or lung cellular injury.

The effects of ALLP and LBFs on morphological and functional integrity of respiratory tract were assessed. Tracheal and lung sections from animals dosed intra-tracheally showed normal morphology indicating that the intra-tracheal dosing procedure did not cause any physical disruption. Moreover, no pathological changes were observed in the trachea and in the lung (Figure 7.8 and 7.9) over the time course of experiments.

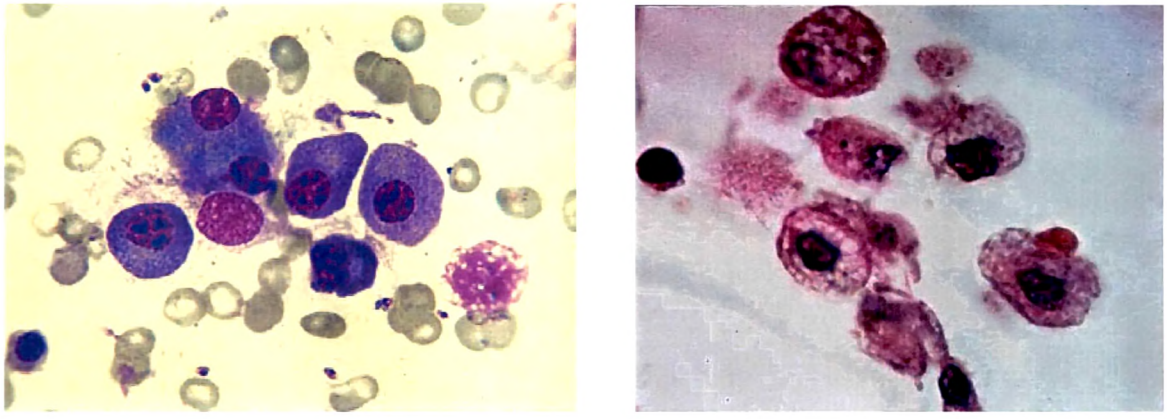
Table 7.4 Lung inflammatory parameters (Mean±S.D, n=3)

Parameter	*Sham operated*	*TB-LBF*	*TB-ALLP*	*AMK-LBF*	*AMK-ALLP*
Total cells (x 10^6)	6.8 ±2.12	5.2 ±1.98	5.7 ±1.92	4.92 ±1.14	5.07 ±1.53
Macrophages(x 10^6)	1.47 ±0.68	0.92 ±0.53	0.94 ±0.41	1.1 ±0.38	0.90 ±0.32
Neutrophils(x 10^6)	2.31 ±1.12	1.14 ±0.73	2.22 ±1.06	1.34 ±0.77	2.09 ±0.88
Eosinophils(x 10^6)	2.69 ±1.44	1.88 ±0.76	2.26 ±0.96	2.22 ±0.87	2.36 ±1.11
Total proteins (mg)	23.45 ±1.64	21.87 ±2.21	24.23 ±2.40	26.28 ±3.64	25.62 ±4.16
AP(nmol of 4-nitrophenol produced min^{-1} mg^{-1} protein)	16.24 ±0.57	15.87 ±0.67	15.16 ±0.74	14.69 ±0.86	15.24 ±1.16
LDH (nmol of NADH oxidized min^{-1} mg^{-1} protein)	102.2 ±3.64	97.25 ±4.22	98.16 ±3.64	100.31 ±3.57	98.76 ±4.73



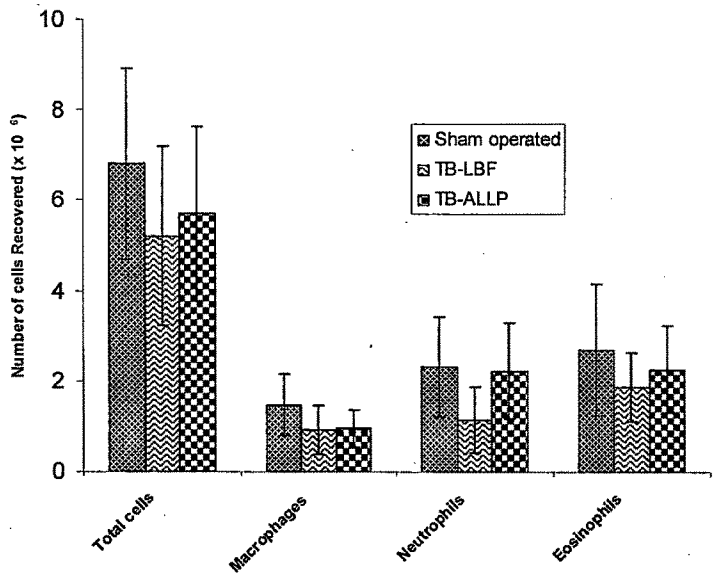
(A) **(B)**

Figure 7.4 **Photomicrographs of alveolar macrophages engulfed of TB(A) and AMK(B) LBF.**

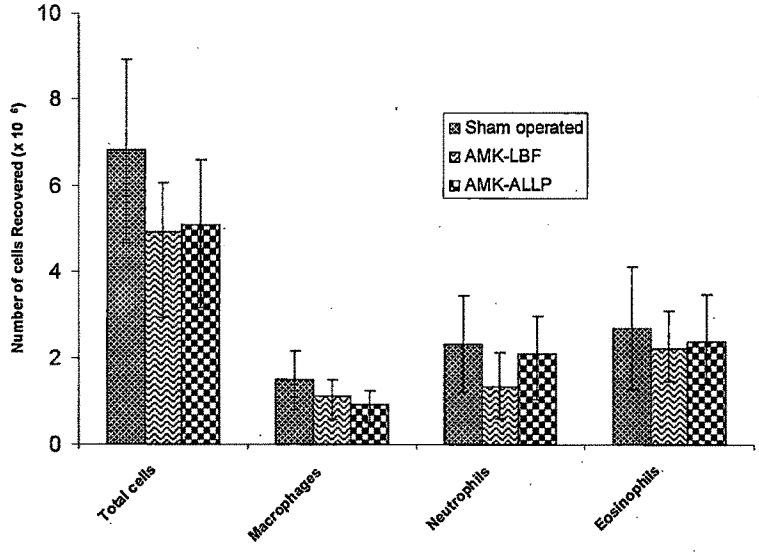


(A) **(B)**

Figure 7.5 **Photomicrographs of representative alveolar macrophages engulfed of TB(A) and AMK(B) ALLP**

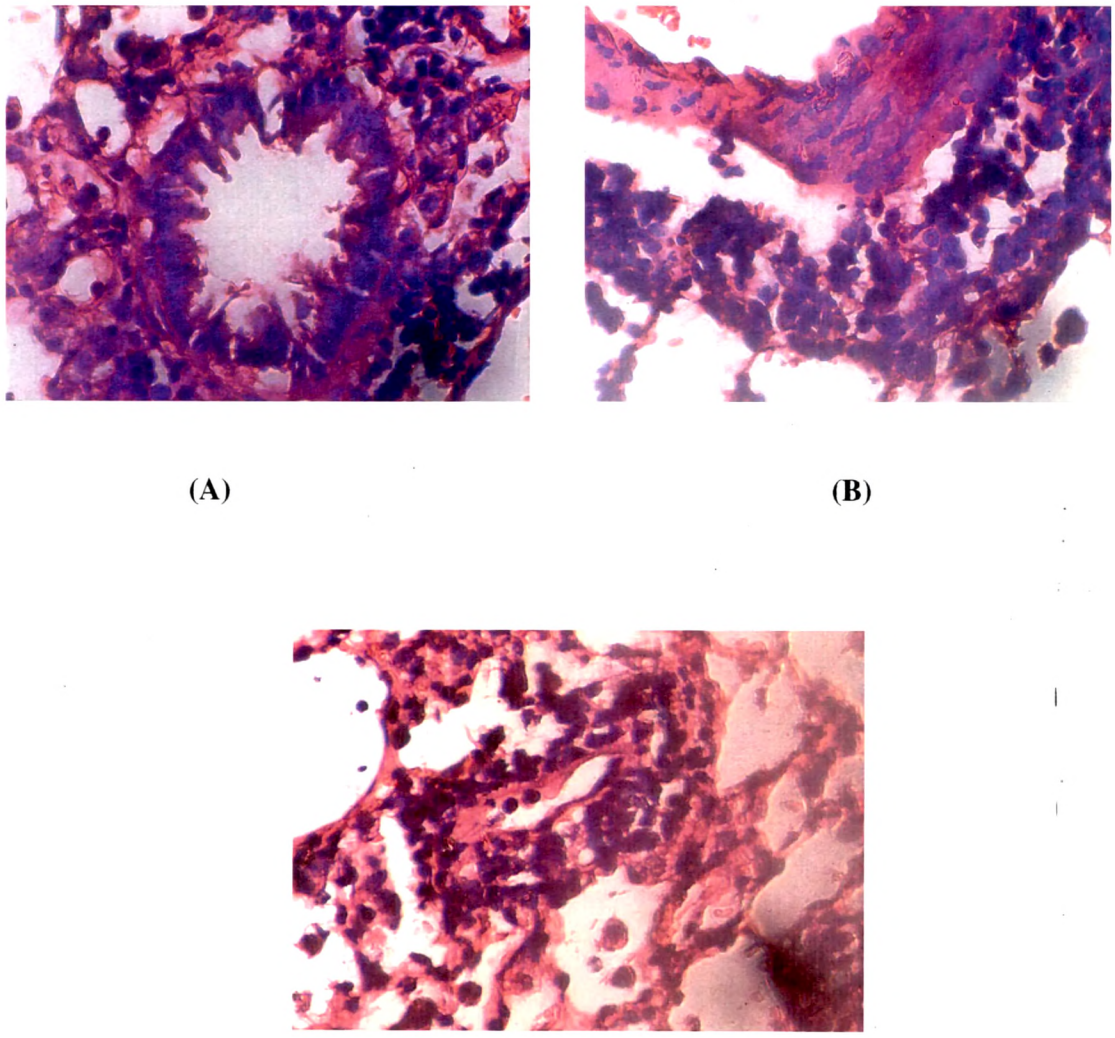


(A)



(B)

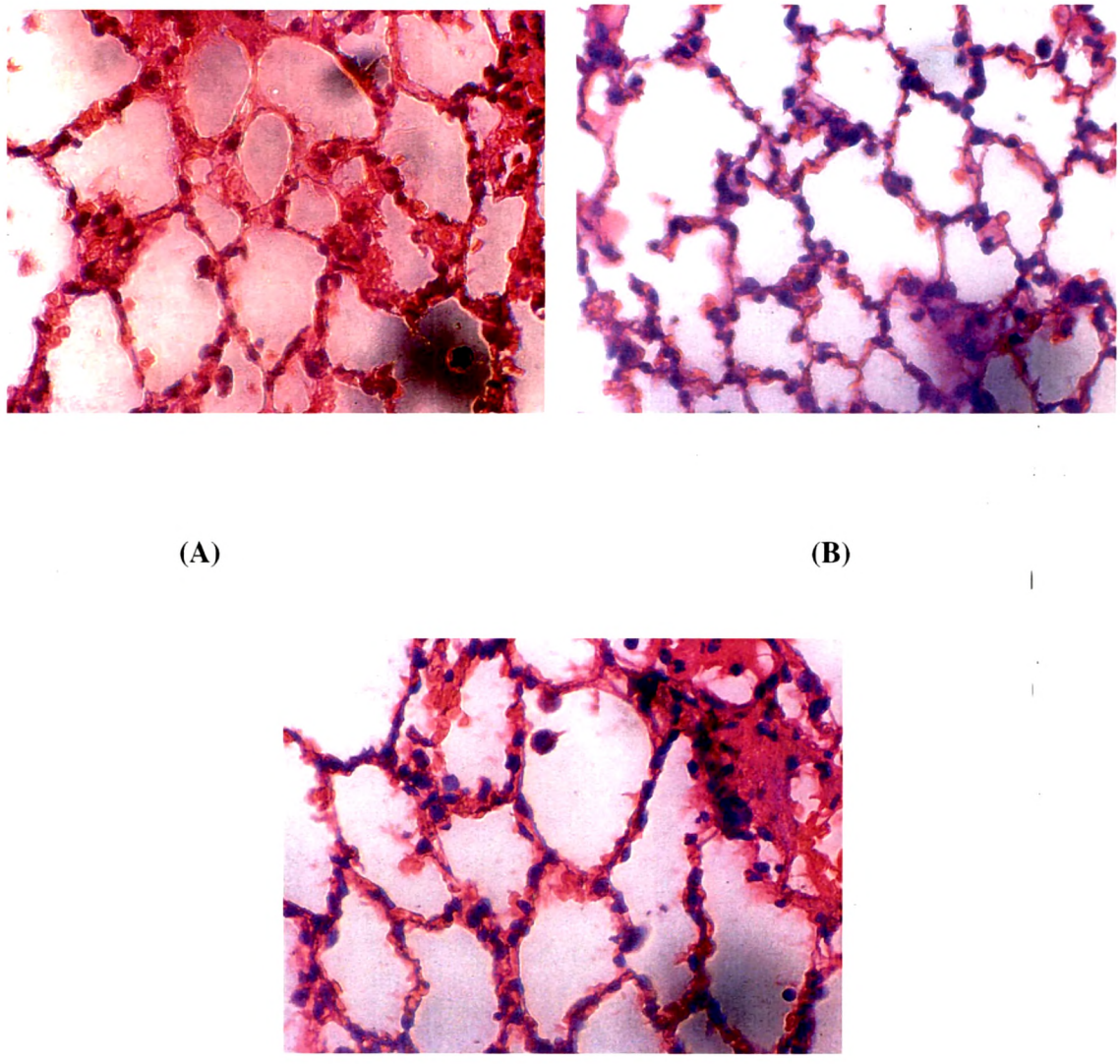
Figure 7.6 Cell recovery from bronchoalveolar lavage fluid administered with TB (A) and AMK(B) formulations (Mean ± S.D; n=3)



(A) **(B)**

(C)

Figure 7.7 **Haemotoxylin and eosin staining of representative tracheal sections following assisted ventilation (A) and intra tracheal administration of ALLP (B) and LBFs (C)**



(A) **(B)**

(C)

Figure 7.8 **Haemotoxylin and eosin staining of representative lung sections following assisted ventilation (A) and intra tracheal administration of ALLP (B) and LBFs (C)**

7.4 REFERENCES

Bodor N., and Buchwald P. Retrometabolism-based drug design and targeting. In: Burgrr's medicinal chemistry and drug discovery. Vol. 2: drug discovery and drug development (Eds Abraham D.). Wiley, New York. 2003.

Edwards D. A., Hanes J., Caponetti G., Hrkach J., Ben-Jebria A., Eskew M.L., Mintzes J., Deaver D., Lotan N., and Langer R. Large porous particles for pulmonary drug delivery. Science. 1997;276: 1868–1871.

Edwards D., Ben-Jebria A., and Langer R.Recent advances in pulmonary drug delivery using large porous inhaled particles.J.Appl.Physiol.1998;84:379-385.

Gupta P.,Qiu Y., and Adjei A.,Pulmonary delivery of 5-lipoxigenase inhibitor Abbott-85761 in beggele dogs.Int. J. Pharm. 1997; 147: 207-218.

Krenis, L. J., and Strauss, B. Effect of size and concentration of latex particles on respiration of human blood leucocytes., Proc. Soc. Exp. Med.1961;107:748-750.

Karlowsky J. A., Zelenitsky S. A., and Zhanel G. G. Aminoglycoside adaptive resistance. Pharmacotherapy 1997; 17:549–555.

Kawaguchi H.,Koiwai N., Ohtsuka Y.,.Miyamoto M., Sasakawa S. Phagocytosis of latex particles by leucocytes. I. Dependence of phagocytosis on the size and surface potential of particles. Biomaterials.1986; 7 (1): 61-66.

Menache M. G., Miller F. J., and Raabe O. G. Particle inhalability curves for humans and small laboratory animals. Ann. Occup. Hyg. 1995;39:317–328.

Vogelman B. and Craig W. A. Kinetics of antimicrobial activity. J. Pediatr. 1986; 108:835–840.

Moss D. W., Acid phosphatases. In Methods of Enzymatic Analysis (Ed H. V. Bergmeyer).Verlag Chemie, Weinheim.1984; 4: 90–106.

Neer R. M., Arnaud C. D., Zanchetta J. R., Prince R., Gaich G. A., Reginster J. Y., Hodsman A.B, Eriksen E. F., Ish-Shalom S., Genant H. K., Wang O., and Mitlak B. H. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N. Engl. J. Med. 2001;344: 1434–1441.

Patton J. S., Trinchero P., and Platz R. M. Bioavailability of pulmonary delivered peptides and proteins: α-Interferon, calcitonins and parathyroid hormones. J. Contr. Rel. 1994; 28:79–85.

Rudt, S. and Muller, R. H. In vitro phagocytosis assay of nano- and microparticles by chemiluminescence. I. Effect of analytical parameters, particle size and particle concentration. J. Contr. Rel.1992; 22: 263-271.

Smith S.J., and Bernstein J.A. Therapeutic uses of lung aerosols.In: Inhalation aerosols (Eds Hicky A.J.), Marcel Dekker,New York.1996;233-269.

Schentag J. J., Birmingham M. C., Paladino J. A., Carr J. R., Hyatt J. M., Forrest A., Zimmer G. S., Adelman M. H., and Cumbo T. J. Nosocomial pneumonia, optimizing antibiotics other than aminoglycosides is a more important determinant of successful clinical outcome, and a better means of avoiding resistance. Semin. Respir. Infect. 1997;12:278–293.

Vanbever R., Mintzes J., Wan J., Nice J., Chen D., Batycky R., Langer R., and Edwards D. A. Formulation and physical characterization of large porous particles for inhalation. Pharm. Res. 1999; 16: 1735–1742.

Vale´Rie C., Francis V., Bernard U., Ve´Ronique P., and Vanbever R. Impact of Formulation and Methods of Pulmonary Delivery on Absorption of Parathyroid Hormone (1–34) from Rat Lungs. J. Pharm. Sci. 2004;93:1241–1252.

Wotton I. D. P., Enzymes in blood. In Microanalysis in Medical Biochemistry. Churchill, London. 1964; 101–118.