Chapter 2

Interaction of epithelial cells and macrophage cell lines with BCG
Air borne pathogens may reach lung alveoli and interact with two types of cells that are present in alveoli. These are epithelial cells and alveolar macrophages. Nature of interactions between pathogen and epithelial cells/macrophages is not clearly understood. Macrophages being phagocytic cells are expected to engulf pathogens. However whether epithelial cells can also internalized pathogens and if they do, the mechanisms their of, is not known. We used BCG as a model pathogen and compared \textit{in vitro} effects of exposure to BCG on the survival and growth of lung epithelial (LA4) and macrophage (MH-S) cell lines in culture. Uptake of BCG by MH-S macrophages and LA4 epithelial cell lines was also compared.

\textbf{Effect of intact BCG and BCG sonicate on LA4 and MH-S proliferation and recovery in culture}

Growth kinetics of LA4 and MH-S cells in culture was studied. Results in Figure 11 (A & B) show that both cell lines doubled their numbers within a period of 72 hrs. In presence of BCG (MOI 1:1 or 10:1) LA4 cells had a significantly higher growth rate than the control untreated LA4 cells. At higher MOI 100:1, the recovery of LA4 was not altered (Figure 11 Panel A). In contrast at all concentrations of BCG, growth of MH-S cells was significantly inhibited at all time point (Figure 11, panel B). The epithelial cell line actually seems to proliferate faster in presence of BCG at MOI (1:1 and 10:1). These results indicate that BCG may have toxic effect on macrophage cell line but not on the epithelial cell line.

Result in Figure 12 further show that the effect of BCG sonicate on cell numbers of LA4 and MH-S cell lines in culture, was similar to what was seen with intact BCG cells. These results indicate that live BCG are not needed for the positive effect of BCG on LA4 cells, since BCG sonicate were equally effective in this respect.

\textbf{Staining of BCG with fluorescent DiI C18 dye}

Effect of BCG on cell numbers could be a consequence of uptake of BCG by epithelial and macrophages cell lines. To study cellular uptake, BCG cells were labelled with a fluorescent dye (DiI C18), so that the uptake of the fluorescenated bacteria by epithelial cells and macrophages could be examined flowcytometrically. BCG cells were labelled with DiI C18 dye as described in methods. Results in
Figure 11: Effect of BCG sonicate (sBCG) on the growth of mouse alveolar lung epithelial cell line LA4 (panel A) and mouse alveolar macrophage cell line, MH-S (panel B) in culture. LA4 and MH-S cells (2.5x10^4/ml/well) were cultured in 48-well culture plates. After overnight culture, cells were infected with BCG at different doses. After 24, 48 and 72 hrs, cells in culture wells were washed, detached by trypsinization and suspended in trypan blue solution in PBS. Recoveries of viable cell numbers were assessed by cell counting using a hemocytometer. Each point represents Mean ±SEM values obtained from 3 replicate assay wells. *p < 0.05 for difference between control and BCG treated cell cultures.
Figure 12: Effect of BCG on the growth curve of cultured mouse alveolar lung epithelial cell line LA4 (panel A) and mouse alveolar macrophage cell line, MH-S (panel B). LA4 and MH-S cells (2.5x10⁴/ml/well) were cultured in 48-well culture plates. After overnight culture, cells were infected with BCG sonicate at different doses. After 24, 48 and 72 hrs cells in culture wells were washed, detached by trypsinization and suspended in trypan blue solution in PBS. Recoveries of viable cell numbers were assessed by cell counting using a hemocytometer. Each point represents Mean ±SEM of values obtained from 3 replicate assay wells. *p < 0.05 for difference between control and sBCG treated cells in culture.
Results

Figure 13, panel A show that unstained BCG have negligible fluorescence. After binding with Dil C18 dye however 82% of BCG cells were labelled with fluorescence. (Figure 13, panels B and C).

BCG uptake by epithelial and macrophage cell lines

Under fluorescence microscope Dil C18 coupled BCG were seen to have bright red fluorescence. In order to asses the uptake of BCG by epithelial and macrophage cell lines, Dil C18 tagged BCG was added to A549, TC-1, LA4 and MH-S cell cultures. At different time points culture cells were harvested and examined on FACS. Cells that had internalized fluorescence tagged BCG could be distinguished from the cells without BCG. Flowcytometric analysis of cultured A549, LA4, TC-1 epithelial cells and MH-S macrophages incubated for different time periods with fluorescently tagged BCG are shown in Figure 14. These results clearly indicate that with the progression of time, increased number of culture cells became associated with fluorescence coupled BCG. This association of BCG with cultured cells could result from an active internalization of BCG or could be due to adherence of BCG to the culture cells. At all time points a significantly larger proportion of MH-S macrophages were found to be associated with BCG as compared to various lung epithelial cell lines. More quantitative time kinetics of uptake/association of BCG with different cell lines have been shown in Figure 15. These results indicate that there is a time dependent uptake/association of BCG with all types of cells. However the rate of uptake of BCG was substantially greater for MH-S macrophages as compared to epithelial cell lines.

Effect of silica on LA4 and MH-S

Effect of microfine crystalline silica particles on cell recovery was examined using LA4 and MH-S cells in culture. Results in Figure 16 (panel A&B) indicate that, all doses (10, 50, 100 µg/ml) of silica particles inhibited significantly the growth of LA4 as well as MH-S cells. The inhibition was almost complete at 50 and 100 µg/ml concentration of silica particles. At 100 µg/ml of silica, recovery of LA4 and MH-S cells fell by approximately 52% and 65% respectively at 24 hrs, time point. These results indicate that microfine crystalline silica particles are toxic to LA4 and MH-S cell lines.
Figure 13: BCG were grown in Sauton's medium as shaken culture. BCG were suspended and incubated with Dil C18 dye at 10 μM. The samples were then incubated in dark at 37°C for 2 hrs. The bacterial pellet was washed twice with PBS. The stained BCG were analyzed on flowcytometer. The horizontal line on X-axis indicates the position of gate determined by using control cells. Unstained BCG (Panel A), Dil C 18 stained BCG (Panel B) and overlay of isotype control and stained BCG (Panel C).
Figure 14: Uptake of Dil C18 stained BCG by epithelial and macrophage cell lines. 0.2x10^6 cells/ml/well were seeded in 24 well plate. After 6 hrs A549, LA4, TC1 and MH-S cells were infected with Dil C18 stained BCG. At different time intervals cells were harvested by trypsinization, washed with PBS, fixed in 2% paraformaldehyde and examined on a flowcytometer.
Figure 15: Uptake of DiI C18 stained BCG by epithelial and macrophage cell lines. 0.2x10^6 cells/ml/well were seeded in 24 well plate. After 6 hrs A549, LA4, TC1 and MH-S cells were infected with DiI C18 stained BCG. At different time intervals cells were harvested by trypsinization, washed with PBS and fixed in 2 % paraformaldehyde. Uptake of stained BCG by cell lines were analyzed on flowcytometer. Each point represents Mean ±SEM of values obtained from 3 replicate assays.
Figure 16: Effect of silica on the growth of mouse alveolar lung epithelial cell line LA4 (panel A) and mouse alveolar macrophage cell line, MH-S (panel B) in the culture. LA4 and MH-S cells (2.5x10⁴/ml/well) were cultured in 48-well culture plates. After overnight culture, cells were treated with silica at different doses. After 24, 48 and 72 hrs cells in culture wells were washed, detached by trypsinization and suspended in trypan blue solution in PBS. Recoveries of viable cell numbers were assessed by cell counting using a hemocytometer. Each point represents Mean ± SEM of values obtained from 3 replicate assay wells. *p < 0.05 for difference between control and silica treated cell cultures.
Basal Nitric Oxide (NO) secretion and effect of silica, LPS and BCG

Interaction of pathogen with macrophages is known to cause the release of Nitric Oxide (NO) by macrophages (MacMicking et al, 1997). It was of interest to see, if BCG could also induce NO in epithelial cells. Bacterial LPS a known potent inducer of NO secretion by macrophage cells was used as a positive control. Release of NO was examined in LA4 epithelial cells and MH-S macrophages in response to LPS and BCG. Results in Figure 17 (panel A) show that control LA4 cells release very low levels of NO (less than 1 μM) and that was not further augmented by LPS or BCG.

Control MH-S cells also secreted very low levels of NO, but in this case both LPS and BCG markedly augment the secretion of NO by MH-S cells after 24 and 48 hrs (Figure 17, panel B). Addition of silica particles resulted in a marginal decline in NO secretion by BCG or LPS stimulated LA4 cells. No effect of silica was seen on LPS and BCG induced NO secretion by MH-S cells (Figure 17).
Figure 17: Effect of silica, LPS and BCG on nitric oxide production on LA4 (panel A) and MH-S (panel B) cell lines. LA4 and MH-S cells were seeded at density of $25 \times 10^4$ / ml/well in 24 well plate. After overnight culture cells were treated with silica (50 $\mu$g/ml), LPS (10 $\mu$g/ml) and BCG ($25 \times 10^5$ cfu) individually or in combination. Nitric oxide concentration was measured in culture supernatants at the 24 and 48 hrs. Each point represents Mean ± SEM of values obtained from 3 replicate assays. *p < 0.05 effect of LPS and BCG over control.