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An approach for studying the mediators of pathogenesis in *Mycobacterium tuberculosis*

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Abstract. ***Mycobacterium tuberculosis*** **is an example of an intracellular pathogen that mediates the disease state through complex interactions with the host's immune system. Not only does this organism replicate in the hostile environment prevailing within the infected macrophage, but it has also developed intricate mechanisms to inhibit several defence mechanisms of the host's immune system. It is postulated here that the mediators of these interactions with the host are products of differentially expressed genes in the pathogen. B and T cell responses of the host are hence to be used as tools to identify such gene products from an expression library of the** ***Mycobacterium tuberculosis*** **genome. The various pathways of generating a productive immune response that may be targeted by the pathogen are discussed.**

Keywords. ***Mycobacterium tuberculosis;*** **macrophages; immune suppression.**

1. Introduction

A report published by the World Health Organization in 1992 (Bloom and Murray 1992) stated that tuberculosis (TB) was the leading cause of death among all infectious diseases. It is estimated that there are 8 million new cases and 2·9 million deaths from this disease annually. One third of the world's population harbours *Mycobacterium tuberculosis* and is therefore at risk for infection. In the developing world, TB accounts for 6·7% of all deaths. In the advanced countries, the steady decline in the incidence of TB since 1882 has shown a reversal over the last ten years. Further, our capability to control the disease has been seriously threatened by the ominous emergence of drug resistant TB. One third of all cases in New York city in 1991 was resistant to one or more drugs. Among patients suffering from multidrug resistant tuberculosis (MDR-TB), the case fatality is as high as 60%, equivalent to untreated TB. *M. tuberculosis* has been found to be the most common opportunistic infection in AIDS patients. In fact, it can be considered as a sentinel disease for AIDS since it is often the first indication of HIV infection. So today, tuberculosis remains a global health problem, in spite of 4 decades of availability of antituberculosis drugs and the widespread vaccination with *Mycobacterium bovis* BCG.

What are the underlying reasons for this stalled progress?

(i) *M. tuberculosis* is a daunting organism to work with. The bacilli divide very slowly—unlike *Escherichia coli* which has a doubling time of 20 min, *M. tuberculosis* divides once every 24 h in favourable conditions. One has to therefore wait 3 to 4 weeks to obtain a colony.
(ii) Mycobacteria have a waxy coat made up of several complex lipids and carbohydrates that renders them impermeable to most drugs. This also makes it extremely

difficult to obtain biological macromolecules such as nucleic acids and proteins from these organisms.
(iii) Due to its pathogenicity and easy transmission by aerosols, a very high level of containment is required to carry out experiments on this organism, which is expensive and not widely available.
(iv) Most importantly, mycobacterial genetics continued to be a poorly developed area. As a result, methods and vectors for mycobacterial gene transfer as well as genetic selection methods have come into existence only in the last 5 years.

The disease process reveals extensive interplay between the pathogen and the immune system of the host. To the immunologist, tuberculosis presents a strange paradox. On the one hand, a preparation of mycobacterial antigens stimulates vigorous immune responses. All of us who have used Freund's adjuvant in immunization will be familiar with its adjuvanticity and ability to enhance recruitment of T and B cells very efficiently. On the other hand, a patient suffering from active infection shows severe depression of T cell responses (Daniel *et al* 1981; Ellner *et al* 1990), as evidenced by a complete loss of skin test reaction to mycobacterial antigens (PPD). The distinction here appears to be dead versus live mycobacteria. The T cell energy is a reversible phenomenon and chemotherapy with the attendant reduction in bacterial load restores skin test reactivity to PPD.

We therefore infer that live mycobacteria during growth produce antigens that down regulate or suppress the immune response whereas killed cells lack the suppressive factors and therefore only manifest their immune stimulatory affect. This therefore led several investigators to search for these so called immune system modulating antigens. The initial attempts were made using the only reagents then available, namely antibodies raised to preparations of mycobacterial antigens. Advances in recombinant DNA technology led to construction of a genomic DNA expression library of *M. tuberculosis* (Young *et al* 1985), which has been extensively screened using these antibodies as well as several monoclonal antibodies to specific antigens. Several of the antigens thus identified turned out to belong to the family of stress proteins (Shinnick *et al* 1988; Garsia *et al* 1989; Kong *et al* 1993).

T cell suppression seen even in the periphery led some researchers to believe that secreted antigens may be the prime mediators of suppression. The literature today on mycobacterial antigens and the immune response elicited by them is a compendium of the B and T cell responses elicited by these antigens, mostly in mice and occasionally in humans, as well as their effects on the production of a battery of lymphokines (Barnes *et al* 1993; Boom *et al* 1990). However, not one of these antigens have turned out to be a successful candidate for eliciting protective immunity. One striking aspect of these studies is that all the antibodies used in these screens were raised to antigens obtained either from killed bacillary extracts or bacilli grown *in vitro.* In addition, the strains of *M. tuberculosis* used were not virulent field isolates, but standard laboratory strains such as the virulent H_{37} R_v and its avirulent counterpart $H_{37}R_a$.

We have therefore used immune serum and immune cells from tuberculosis patients to identify immunogenic antigens elaborated by the organism. The immunogenicity of the tuberculosis antigens when administered as pure preparations is likely to be different from the immunogenicity of these antigens when they are elaborated within the infected host during an active infection. The B and T cells from tuberculosis patients

and healthy contacts are therefore most likely to be able to identify the antigens responsible for mediating pathogenesis and protective immunity, respectively.

2. Materials and methods

2.1 *Culture of macrophages*

Lungs obtained from guinea pigs were washed extensively with phosphate buffered saline to shed macrophages. The cells were washed once with phosphate buffered saline and cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) and 5×10^{-5} M *ß*-mercaptoethanol in a 5% CO_2 atmosphere.

2.2 *Growth of M. tuberculosis*

The avirulent strain H_{37} R_a as well as a high virulent field isolate var 83949 were grown in Middlebrook 7H9 broth containing 0·5% albumin and 0·05% Tween 80. After growth for 7 to 10 days in a rotary shaker, clumps were allowed to settle by standing. The supernatant containing predominantly single cells were pelleted by centrifugation, washed with RPM1-1640 and suspended in RPMI-1640 containing 10% FBS. Aliquots were frozen at — 70°C.

2.3 *Infection of macrophages with M. tuberculosis*

Monolayers of macrophages were infected with the bacteria at a multiplicity ranging between 10 and 30 for a period of 4 h. Unadsorbed bacilli were removed by washing and the cells were replaced in the incubator and cultured for up to 10 days. Zeihl-Neelsen staining and Leishman's staining was according to standard procedures.

2.4 *Serum*

Blood was obtained from patients diagnosed to be suffering from *M. tuberculosis* infection by Ziehl-Neelsen staining of sputum samples, and used as source of serum
Neutral red staining of macrophages was for 5 min at a final concentration of 0·01 %.

2.5 *Screening of recombinant plaques*

Plaques obtained on a lawn of *E. coli* XLl-Blue cells were induced by overlying with IPTG-impregnated nitrocellulose membranes. TB patient serum at a dilution of 1:100 was used followed by protein A-horse radish peroxidase. Colour was developed using diaminobenzidine as substrate.

3. Results

When we began our efforts at understanding mycobacterial pathogenesis, we started with the following major assumption:

During an active infection within the susceptible host, *M. tuberculosis* must turn on expression of a set of genes whose products are required for mediating pathogenesis and suppressing host imunity.

It would follow from the above assumption that these antigens would not be present in a preparation obtained from a culture of *M. tuberculosis* grown *in vitro.* Vaccination with attenuated or killed *M. bovis* BCG cannot be very effective in protection since the vaccinating antigen lacks the above mentioned differentially expressed gene products and specific immunity to these antigens may be essential for conferring protection. We know today that the worldwide immunization programme using BCG was a near failure except in Western European countries where prior exposure of the population to mycobacterial antigens was very low.

The above assumption forms the basis for the questions we addressed relating to the development of pathogenesis in *M. tuberculosis.* Each pathogen has evolved over hundreds of thousands of years along with its specific host. This coevolution has afforded ample opportunity for the development of very complex and intimate interactions between the two. Vertebrates have developed a very sophisticated immune system and invading organisms have discovered numerous ways to circumvent its powerful mechanisms, and in some cases, even to coopt its components for their own purposes.

In the case of many viruses as well as in certain parasites the mechanisms adopted by the pathogen to subvert the host's immune responses have been elucidated in depth. However, in the case of *M. tuberculosis,* although the gross phenomenon of immune suppression is evident, the specific mediators of the effect and their mechanism of action are yet to be identified. One exception, perhaps is lipoarabinomannan (LAM), a component of the mycobacterial cell wall, which has been shown to stimulate specific gene expression (Roach *et al* 1993) in host cells.

We decided to use the B and T cells of the host's immune system as reagents to search for these mediators. These cells alone are likely to have seen these differentially expressed molecules and therefore should have been specificially primed to these antigens. In addition, such gene products are likely to be expressed only within infected macrophages. We turned to the animal model guinea pig which is best suited for studying the pathogenesis of *M. tuberculosis.* Guinea pigs are uniformly susceptible to tuberculosis and the disease progression is rapid with involvement of spleen, liver and lungs.

We obtained pulmonary alveolar macrophages from guinea pigs. Lungs were washed with saline extensively to shed the loosely attached macrophages. These cells could then be maintained in culture for 4 to 6 weeks during which time they do not divide but merely differentiate into large cells. This length of time is sufficiency for experiments involving intracellular growth of *M. tuberculosis* which takes 2 to 3 weeks to grow to sufficient numbers. Figure 1A shows these cells soon after they are obtained from the lung. To establish their identity as macrophages, we used the neutral red uptake assay for phagocytic cells, shown in figure 1B. The macrophages instantaneously concentrate the dye and appear stained red. In addition to macrophages, the pulmonary lavage contained small numbers of lymphocytes which can also be seen on leishman staining (figure 2A). Figure 1C shows the appearance of these same cells after 2 weeks in culture, by which time the cells have fanned out into large macrophages. Figure 1D shows neutral red stained differentiated macrophages.

We also obtained lung macrophages from infected animals. A high virulent field isolate of *M. tuberculosis* was used for these experiments. Guinea pigs were infected

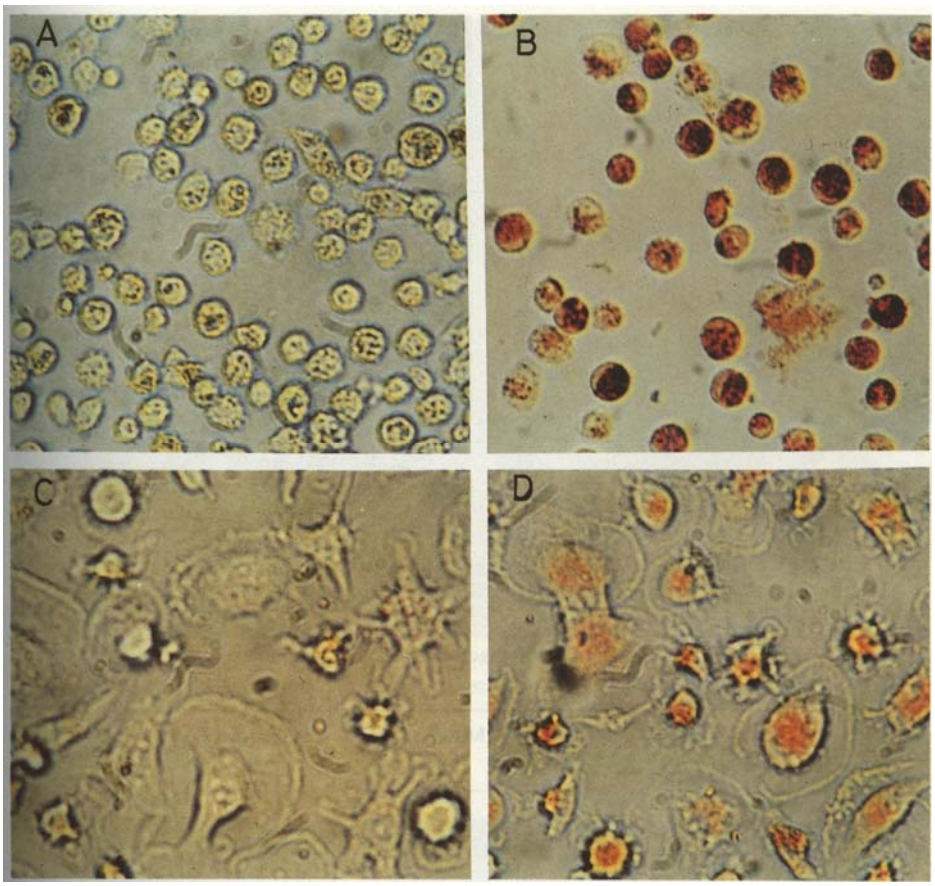

Figure 1. Guinea pig pulmonary macrophages. **(A)** Pulmonary lavage cells 6 hours after harvest. **(B)** Neutral red stained cells from A. **(C)** Pulmonary lavage cells 1 week after harvest. **(D)** Neutral red stained cells from C.

intramuscularly with 10^7 bacilli and 6 weeks later, the animals were sacrificed. We found the lungs of such animals densely studded with tubercles. To our surprise, the lavage of infected lungs contained a very large proportion of lymphocytes (table 1). This is clearly evident on leishman staining which is shown in figure 2B. This indicates that the active replication of the organism in the lung recruits lymphocytes that now infiltrate into the site of infection. However, intracellular bacilli were found only in an insignificant proportion of macrophages. This was the case regardless of the time point after infection at which macrophages were obtained from the lungs. At times varying from 14 to 42 days post infection, during which period total numbers of bacilli in the lung increased exponentially, we found little variation in the percentage of cells carrying intracellular bacilli. This indicates that most of the bacilli replicate extracellulary in the tubercles and only a small proportion grows intracellularly within macrophages.

We therefore resorted to *in vitro* infection of macrophages to obtain a sizeable proportion of infected cells. Figure 3 shows intracellular bacilli growing within infected macrophages. It is possible to achieve infection of 90% or more macrophages by using a multiplicity of about 40 bacilli per cell. This system will now serve as a source of

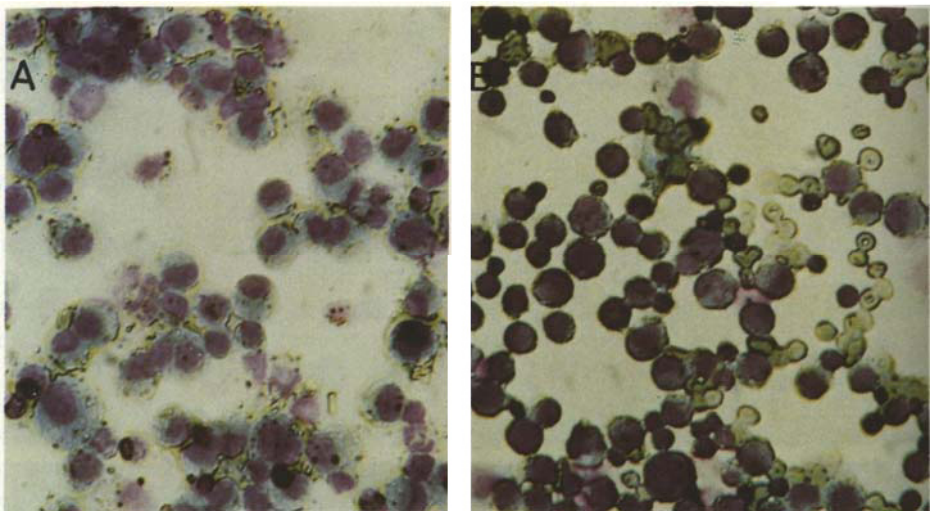

Figure 2. Leishman stained guinea pig lung lavage cells. **(A)** Pulmonary lavage cells from healthy guinea pigs. **(B)** Pulmonary lavage cells from *M. tuberculosis* infected guinea pigs.

Table 1. Distribution of macrophages and lymphocytes in guinea pig lung lavage.

	Macrophages (%)	Lymphocytes (%)
Healthy $n = 6$	90–96	4–10
M. tuberculosis infected	53	47
	61	39
	63	37
	85	15
	58	40
	68	31
	73	25
	89	9

mycobacterial antigens that would be differentially expressed during intracellular growth.

Serum obtained from patients suffering from tuberculosis was used as a tool to identify B cell stimulating antigens of *M. tuberculosis.* A genomic DNA expression library of a high virulent field isolate of *M. tuberculosis* (Amara Rama Rao and Satchidanandam Vijaya, unpublished results) was probed with pooled serum from patients. This screen yielded a large number of recombinants expressing mycobacterial antigens. Figure 4 shows two representative recombinant plaques obtained from this library during the course of plaque purification. From among these, genes that encode the differentially expressed antigens will be identified using suitable screening procedures. Differentially expressed T cell stimulatory antigens will also be identified using this expression library.

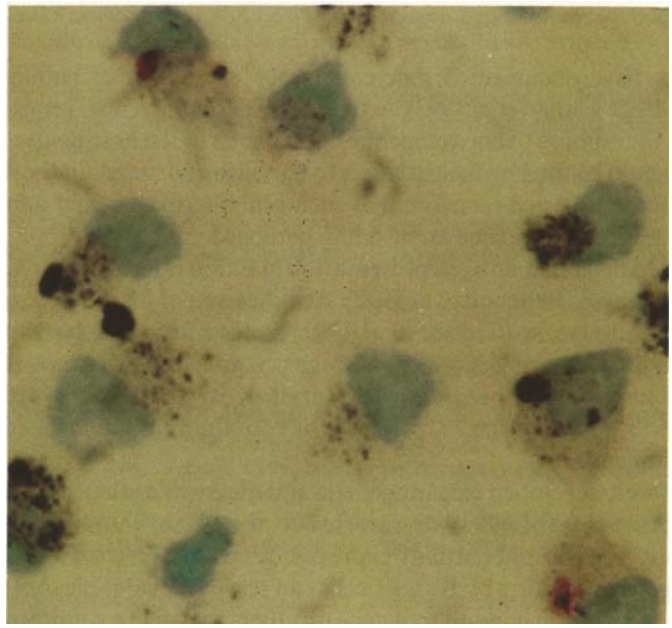

Figure 3. Ziehl Neelsen stained *M. tuberculosis* infected guinea pig pulmonary macrophages.

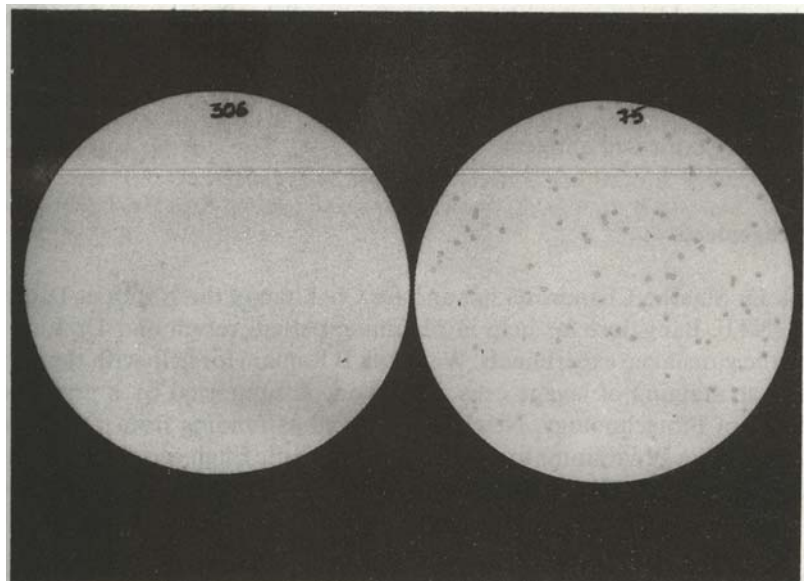

Figure 4. Screening of ZAP-II-*M. tuberculosis* recombinant phage with tuberculosis patient· serum.

Two individual plaques obtained from the first round of screening the library were plated and rescreened with patient serum as described in § 2·5.

4. Discussion

Several reports have documented differential gene expression in pathogenic bacteria (McEvoy *et al* 1990; Mahan *et al* 1993). Activation of such differential genes is presumably brought about in response to environmental cues unique to the host tissues. The products of such genes would be used by the pathogen to establish and maintain the disease state in the host. It is to be expected therefore that such gene products would interfere with the host's normal ability to overcome an invading pathogen.

It has been reported that antigen presentation function is impaired in *M. tuberculosis* infected macrophages (Pancholi *et al* 1993). Acidification of *M. tuberculosis* containing phagosomes fails to occur (Crowle *et al* 1991) as also fusion to lysosomes (Xu *et al* 1994), which is essential for degradation of the phagocytosed organism by lysozomal hydrolases. It has recently been shown (Sturgill-Koszycki *et al* 1994) that selective exclusion of H^+ ATPase in the *M. tuberculosis* containing vacuoles is responsible for the lack of acidification.

For want of well developed elegant genetic systems such as that used for *Salmonella typhimurium* (Mahan *et al* 1993) we resorted to the use of immune cells of infected individuals to screen for differentially expressed genes of *M. tuberculosis*. The specific nature of antigen recognition by B and T cells can serve as a valuable tool in this search However, it is to be borne in mind that gene products that act to suppress immune cells will escape this screening procedure. In order to trap such antigens, it will be necessary to use techniques such as subtractive hybridization of messenger RNA populations.

Macrophages obtained from guinea pig lungs support the growth of *M. tuberculosis in vitro* and is expected to mimic the *in vitro* growth of the organism within the host. Here again, one has to bear in mind that during an active infection process, infected macrophages would interact with other immune cells such as neutrophils and lymphocytes, as well as become the target of several soluble cytokines secreted by other cell populations. Owing to our inability to mimic this complex interplay of immune cells *in vitro,* we also propose to obtain *M. tuberculosis* from infected animal tissues as a source of differentially expressed antigens.

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