Mycobactericidal nature of human monocytes *in vitro*

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Protective immunity in tuberculosis is attributed to destruction and elimination of the pathogen *M. tuberculosis* by activated macrophages. Activation of macrophages is brought about by the lymphokines released by antigen sensitized T lymphocytes. In the past ten years several review articles and research papers have been published on the antimycobacterial activity of macrophages. However convincing evidence of intracellular killing of *M. tuberculosis* was lacking in most of these experiments. An *in vitro* experiment wherein we are able to demonstrate mycobactericidal activity by monocytes could yield valuable information in understanding of tuberculoimmunity and also to resolve immunomodulating agents as an adjunct to chemotherapy.

TUBERCULOSIS is an ancient human scourge that has apparently plagued man ever since human beings emerged as a species on this planet and continues to be an important public health problem world-wide[1]. Throughout the world, tuberculosis is a disease that represents a dynamic balance between man and *Mycobacterium tuberculosis*[2]. Its depredations, especially over the last hundred years have earned it the epithet 'The Captain of all the Men of Death'[1].

Tuberculosis is on the rise again especially among individuals suffering from acquired immunodeficiency syndrome. Kochi and Crawford stressed the size of the tuberculosis problem and aggravating circumstances in which it develops: by the year 2000, an estimated 10 million people will freshly acquire active disease annually, of which one third will die[3]. The continuing increase in mycobacterial disease is mainly due to population growth in areas with poor hygiene and low socio-economic resources, with coincident HIV disease accounting for approximately 14% of the surge by the end of this century[4]. The spread of multi-drug resistant TB strains has become most obvious in AIDS patients who, because of their increased susceptibility to infection and manifestation of disease, represent early indicator cases[5].

M. tuberculosis is a facultative intracellular pathogen that lives and multiplies inside nonactivated macrophages. It has been recognized for some time that immunity to infection by facultative intracellular pathogens is not mediated by antibodies. In case of tuberculosis, the lack of any role for antibody is supported by the fact that either in natural or experimental disease there is no relationship between the amount of antibodies formed and immunity to tuberculosis[6].

Also experimentally investigators have been unable to transfer immunity from immunized animals to non immunized animals by injecting serum that contains antibodies to constituents of tubercle bacillus[6]. Furthermore, it has been shown that immunity to tuberculosis infection can be transferred to non immune animals by using either peritoneal exudate cells or thoracic duct lymphoid cells[7–10].

It is widely believed that cell-mediated immunity and the associated ability of the macrophages to destroy or inhibit the bacillus is all that is required to control pulmonary tuberculosis[11]. The macrophage possesses multiple functions both in the primary and secondary immune responses[12]. Its function as the ultimate expressor of bactericidal (or bacteriostatic) activity against the intracellular pathogen makes it central to our understanding of antituberculosis resistance[13]. Current technologies (both *in vivo* and *in vitro*) for studying the intracellular killing of microbial pathogens are flawed, and we urgently need to develop new quantitative methods for determining the intraphago-lysosomal events leading to bacterial inactivation so that the molecular nature of the mechanisms responsible for limiting the progressive growth of the pathogen within the macrophage can be understood.

In the past ten years, several papers have been published on the antimycobacterial activity of macrophages[14–22]. In these experiments, the macrophage-activating agents ranged from crude lymphokines to recombinant interleukins and immunologically active vitamins like vitamin D and vitamin A. However, convincing evidence of intracellular killing of *M. tuberculosis* was lacking in most of the experiments and the various macrophage activating agents resulted only in achieving bacteriostasis.

In this article the overall information available on the antimycobacterial nature of human monocytes *in vitro* has been briefly reviewed.

Macrophages: the primary defenders against *M. tuberculosis*

The first direct demonstration that macrophages were involved in immunity to tuberculosis was provided by

Lurie[7] in 1942. Using eliciting agents, he induced inflammatory peritoneal exudates in both normal and immunized rabbits. The peritoneal cells (approximately 75% mononuclear in type) were removed from the peritoneal cavities of the rabbits and then washed and mixed with virulent human type tubercle bacilli. Phagocytosis of the tubercle bacilli was allowed to take place for one hour, after which the extracellular bacilli were removed. Normal or immune serum was then added to aliquots of the infected cell suspensions. These infected cell serum mixtures were then injected into the anterior chamber of one eye of rabbits and allowed to incubate their for 10 to 14 days. At the end of this period, the number of tubercle bacilli in the eye of each rabbit was determined by removing the fluid from the iris and making direct counts microscopically. The number of mycobacteria found in the fluid and the iris of those animals implanted with immune cells was usually lower than the tubercle bacilli found in the fluid and iris of rabbits that had been implanted with infected normal cells (not from immunized animals).

The ability to inhibit the proliferation of the bacilli also seemed to be uniquely a property of the phagocytic cells present, since immune serum plus immune cells yielded results similar to those obtained with immune cells mixed with normal serum. However, due to the use of few animals and the variability of the data, the validity of Lurie's conclusions is questionable.

Mechanisms of intracellular killing of mycobacteria

The two important and well-understood mechanisms by which phagocytes eliminate intracellular pathogens are: (i) respiratory burst and (ii) degradation of intracellular pathogen by lysosomal enzymes.

Respiratory burst is a metabolic pathway which is generally absent in normal cells. The function of the respiratory burst is to produce a series of active metabolites which are potent microbicidal agents[14,15].

Characteristic features of respiratory burst are:
(i) increased consumption of oxygen.
(ii) production of oxygen metabolites like superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals and
(iii) metabolism of glucose by hexose monophospate shunt and generation of $NADPH_2$ in parallel.

When macrophage plasma membrane is perturbed respiratory burst is triggered[14–18]. Phagocytosis induces respiratory burst. Respiratory burst can also be induced in macrophages *in vitro* by cytokines like interferon γ (IFN γ) and agents like PMA[19].

Role of oxygen metabolites

The membrane-bound enzyme NADPH oxidase gets activated when the macrophage plasma membrane gets perturbed.

NADPH oxidase reduces oxygen to superoxide. Superoxide molecule undergoes dismutation in the presence of superoxide dismutase to hydrogen peroxide. Hydrogen peroxide is a microbicidal agent, which brings about the intracellular killing of many organisms like leishmania, trypanasoma and *M. microti*[20–23].

Intracellular killing of any pathogen by phagocytes depends on the following conditions:
(i) ability of the pathogen to trigger respiratory burst.
(ii) susceptibility of the pathogen to the microbicidal agents, produced by the phagocytes.

Hydrogen peroxide may also react with superoxide to produce hydroxyl radicals and singlet oxygen. It has been reported by Cohn and Murray that hydroxyl radicals bring about the intracellular killing of *Toxoplasma gondii*[21,24]. The oxygen metabolites released due to respiratory burst are toxic to the host also, therefore, they are immediately detoxified.

Superoxide undergoes spontaneous dismutation to hydrogen peroxide when the pH is acidic. At neutral pH superoxide is dismutated to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is detoxified to water by glutathione peroxidase.

The role of oxygen metabolites in affording protection against mycobacterial infections was first demonstrated by Walker and Lowrie[22]. Mouse peritoneal macrophages after pretreatment with Spleen Cell Growth factor (SCG) *in vitro* were infected with *M. microti*. The SCG-treated activated macrophages were able to bring about the killing of *M. microti* as compared to control macrophages which were not treated with SCG. The intracellular killing of *M. microti* was mediated by hydrogen peroxide. This was confirmed by the addition of catalase (H_2O_2 scavenger) to the infected macrophage cultures.

However Swamy[25] has demonstrated that the killing of *M. tuberculosis* by human monocyte derived macrophages (HMDM) *in vitro* is not mediated by H_2O_2. Earlier reports emphasize the major role played by hydrogen peroxide as a mycobactericidal agent, this means in tuberculosis patients, the macrophages should be defective with respect to hydrogen peroxide production. In this study hydrogen peroxide was assayed from peripheral blood HMDM from both tuberculosis patients and normals. There was no difference between HMDM from tuberculosis patients and normals with respect to H_2O_2 production. Peripheral blood HMDM *in vitro* may not represent tissue macrophages to the fullest extent. Therefore the same experiment was repeated with peritoneal macrophages from patients suffering from abdominal tuberculosis and alveolar macrophages from paucibacillary tuberculosis patients. Peritoneal macrophages and alveolar macrophages from non tuberculosis

individuals were treated as control. Surprisingly, it was observed that tissue macrophages form tuberculosis patients produced much higher levels of H_2O_2 than when compared to control macrophages, which clearly indicates that the intracellular killing of *M. tuberculosis* is not mediated by hydrogen peroxide.

Interferon γ as macrophage activating agent

The classical macrophage activating factor, interferon γ (IFN γ) has multiple immunological functions[26]. This cytokine (predominantly produced by Th1 subset of CD4 positive cells) is known to induce phagosome lysosome fusion and generation of oxygen and nitrogen metabolites. IFN γ is known to activate indoleamine dioxygenase, an enzyme which brings about tryptophan degradation[27]. Depriving the intracellular pathogen from tryptophan is an attempt made by the host cell to check the multiplication of the pathogen[28]. In fact, it has been recently reported that IFN γ in association with other Th1 cytokines inhibit the multiplication of HIV in Th1 cells.

Flesch and Kaufmann[29,30] have shown that IFN γ is capable of inducing respiratory burst in murine bone marrow macrophages *in vitro*. But none of the oxygen metabolites generated due to IFN γ played any role in bringing about the intracellular killing of *M. bovis*. IFN γ no doubt activates the macrophages to bring about the intracellular killing of *M. bovis* but the killing is not mediated by the oxygen metabolites. Probably IFN γ enhance phagosome lysosome fusion which might contribute for the killing.

But even before Kaufmann, Douvas *et al.*[31] showed that IFN γ could (i) activate HMDM to bring about the intracellular killing of leishmania *in vitro* and also activate HMDM to become tumouricidal. On the contrary, IFN γ enhances the multiplication of *M. tuberculosis* inside the HMDM *in vitro*. However gene knock out experiments performed in mouse models, reveal that when the gene for interferon γ was knocked out, the ability of the mutant animal to kill *M. tuberculosis* was completely lost[32]. In fact, nitric oxide-producing capacity was lost and the granuloma was necrotic. When IFN γ was reconstituted into the system by implanting IFN γ producing osmotic pump there was improvement in the immune status of the animal.

IFN γ not having any role in activating the human monocytes to bring about the intracellular killing was also shown by Rook *et al.*[32]. These workers did a comparative study of mouse macrophages and HMDM with respect to the intracellular killing of *M. tuberculosis*. They concluded that mouse macrophages were able to bring about the growth inhibition of *M. tuberculosis* upon treatment with IFN γ whereas IFN γ enhanced the growth of *M. tuberculosis* inside the human monocytes.

Calcitriol as macrophage-activating agent

Vitamin D compounds are important components of the endocrine system being largely responsible for calcium metabolism. But the active member of the group, calcitriol also interacts extensively with the immune system[33]. Calcitriol is synthesized due to hydroxylation of the precursor 25 (OH) vitamin D3 in the kidney. When calcium level in the body goes down, calcitriol is synthesized by the kidney which in turn regulates the calcium metabolism.

In *M. tuberculosis* infection and sarcaidosis the alveolar macrophages upon activation with interferon γ acquires 1 alpha hydroxylase activity and convert 25 (OH) vitamin D3 to 1,25-$(OH)_2$ vitamin D3 (calcitriol). Calcitriol is an immunologically active metabolite of vitamin D3. It plays a major role in inducing TNF α production, giant cell formation and granuloma formation which results in the containment of infection.

Stasis of *M. tuberculosis* in the intracellular environment of HMDM *in vitro* was first demonstrated by Rook *et al.*[34]. HMDM seven days in culture were activated with IFN γ and 1,25-$(OH)_2$ vitamin D3. When calcitriol and IFN γ-treated marcophages were infected with *M. tuberculosis*, the activated macrophages were able to bring about growth inhibition of *M. tuberculosis*. When HMDM were treated with IFN γ alone no stasis was observed.

Oxygen independent mechanism of killing

The degranulation that normally accompanies and follows phagocytosis results in the release of enzymes and other reactive species into the phagocytic vacuole containing the ingested organism. The macrophage population involved in an infection is heterogeneous in its anti-microbial mechanisms in consequence of differences in the degree of local or systemic activation[19]. The various antimicrobial systems of macrophage that are likely to be substantially independent of the influence of oxygen are described below.

Acid

The pH falls within the phagocytic vacuoles in polymorphonuclear neutrophil and macrophage soon after the phagocytosis. This fall in pH may be important in several ways. Some organisms seemingly are sensitive just to acidity itself, and their susceptibility to low pH appears independent of the anion present.

The susceptibility of organism such as tubercle bacillus depends on the nature of the acid. Many organisms including tubercle bacilli although not necessarily killed by a drop in pH at around 5 may have their growth

arrested or slowed and this might allow time for the immune system to be fully activated to kill the organism[19].

Lysozyme

It is not clear if lysozyme plays a significant role in bacterial killing by macrophages. The evidence suggests a digestive rather than lethal role for the enzyme. Moreover, since less than 20% of the enzyme synthesized by the macrophages is retained intracellularly it is probable that its major function is extracellular[19]. Lysozyme was shown to be essentially bacteriostatic to mycobacteria *in vitro*, and very large, perhaps physiologically unrealistic concentrations were required for killing[35].

Iron sequestration

Transferrin (iron-binding found in serum) has been shown to be bacteriostatic to tubercle bacilli. Macrophages have transferrin receptors and sequester iron as ferritin in lysosomes. Although, iron availability can be growth limiting for bacteria in macrophages *in vitro*, nothing is known of the intracellular regulation of the iron availability and its significance in antimicrobial activities of macrophages *in vivo*[19].

Role of nitrogen metabolites in the intracellular killing of pathogens

Since it has been proved beyond doubt that oxygen metabolites do not bring about the killing of *M. tuberculosis*, attention has been focused towards nitrogen metabolites[36].

Nitric oxide has a wide range of biological function. Nitric oxide released from the endothelial cells upon stimulation with bradykinin, acetyl choline and thrombin is known to induce vasorelaxation[37]. Nitric oxide is synthesized from L. arginine by nitric oxide synthase (NOS). The NOS enzyme in the endothelial cells is known as constitutive NOS which is calcium dependent and contribute more for vasorelaxation and platelet disaggregation[38-41].

The role played by NO in bringing about the intracellular killing of pathogen is known very recently. NOS enzyme is also present in monocytes and macrophages. It is known as inducible NOS and is different from constitutive NOS present in endothelial cells. The inducible NOS can be activated by cytokines and not by bradykinin and acetyl choline. Inducible NOS requires tetrahydrobiopterin as a cofactor[42].

It has been recently demonstrated that NO bring about the intracellular killing of *Toxoplasma gondii, Leishmania tropica, Cryptococus neoformans* and *Schistosoma haematobium*[43,44]. The mode of action of NO is as follows: Nitric oxide is known to be a powerful iron chelator[45,46]. Enzymes like, complex 1, complex 2 (which take part in the electron transport system), aconitase (Krebcycle enzyme), ribonuleotide reductase (DNA replication) have Fe^{2+} as prosthetic group. Therefore it is well understood that NO alters the activity of all these enzymes. NO production can be inhibited by various agents like corticosteroids, dexamethasone and arginase. NO activity is inhibited by superoxide dismutase and methylene green.

According to Schneeman[47], nitric oxide synthesis is very inefficient in human monocytes and macrophages *in vitro*. When mouse macrophages, human monocytes and tissue macrophages were exposed to LPS, endotoxin, IFN γ, TNF α, GMCSF and live bacteria, it was observed that mouse macrophages were able to synthesize nitric oxide whereas the human monocytes and macrophages did not synthesize nitric oxide[47]. The failure of human monocytes and macrophages to synthesize nitrogen metabolites *in vitro* could be due to the defect in the nitric oxide synthase. Nitric oxide synthase requires a cofactor tetrahydrobiopterin (THB) which is present at a very low concentration in the human macrophage[47]. When THB was supplemented in culture medium, then also nitric oxide production did not improve. These workers also observed that human monocytes and macrophages *in vitro* consume very less L. arginine from the culture medium than other amino acids.

Tumour necrosis factor alpha as macrophage-activating agent

Tumour necrosis factor alpha in association with other cytokines activates the macrophages to bring about the killing of intracellular pathogens[48]. Dennis[49] has demonstrated 50% intracellular killing of *M. tuberculosis* by HMDM. In this study human peripheral blood monocytes *in vitro* were pretreated with TNFα, IFN γ and calcitriol and then infected with *M. tuberculosis*. Fifty per cent loss in the intracellular viability of *M. tuberculosis* was observed. Human monocytes treated with IFN γ alone were not able to bring about stasis or killing, while monocytes treated with calcitriol alone were able to bring about stasis but not killing. Monocytes treated with TNF α, IFN γ and calcitriol were able to kill 50% of the phagocytosed *M. tuberculosis*[49].

Dennis[50] has also shown that, IL-12 enhanced the cytotoxicity of natural killer cells, which in turn lyse the monocytes infected with *M. tuberculosis*, thereby exposing the *M. tuberculosis* to cytotoxic factors.

Mechanisms adopted by mycobacteria to avoid killing by macrophages

Uptake of bacteria into phagosomes is usually followed

by the fusion of phagosomes with lysosomes and degradation of the bacteria. Original studies by Hart *et al.*[51] indicated that *M. tuberculosis* can inhibit phagolysosomal fusion, possibly through the action of surface sulphatides[52]. *Mycobacterium avium* and *M. leprae* like other intracellular pathogens also inhibit phagolysosomal fusion. Although opsonization of mycobacteria with serum and antibody partially reverses the inhibition of the fusion, virulent *M. tuberculosis* continue to replicate within the fused phagolysosomes, emphasizing that mycobacteria have multiple survival mechanisms[53].

It is generally believed that elimination of *M. tuberculosis* from the tissues of protected individuals depends on destruction of the organism inside the macrophages, that have been activated by the lymphokines secreted by T lymphocytes. The right lymphokine that activates the macrophages and the right *in vitro* conditions to demonstrate the antimycobacterial activity has been a matter of debate for a long time. Though IFN γ has been shown to have protective effect for macrophages against various pathogens and some intracellular bacteria, its non protectiveness against intracellular mycobacteria has been recently confirmed[24,54]. The observations of Crowle *et al.*[55] that 1,25-$(OH)_2$ vitamin D_3 act as an immunomodulator that can reproducibly activate human macrophages to express tuberculoimmunity were further extended by Dennis. He demonstrated that human monocytes upon activation with IFN γ, TNF α and calcitriol kill 50% of the *M. tuberculosis*[49]. Warwick and others have tried to repeat the findings of Dennis under identical *in vitro* conditions but ended up with negative results[56].

From the studies carried out in our laboratory we were able to observe that human monocytes *in vitro* upon activation with different combination of cytokines and activating agents such as LPS, TNF α, IFN γ and PMA did not improve the ability of the monocytes to inhibit the growth and multiplication of *M. tuberculosis* (unpublished data).

Recently, Molloy *et al.*[57] highlighted that control of tuberculous infection occurs in a granuloma and is intimately associated with the accumulation, activation and death of mononuclear leukocytes. They pointed out that cell death could be due to necrosis or apoptosis and provided further data to show that only during apoptosis (induced by ATP), but not necrosis, of chronically infected cells resulted in a 60–70% loss in the viability of intracellular BCG. They assumed that observations made on one mycobacterium species were relevant to the others. On the contrary, when we treated *M. tuberculosis* and *M. smegmatis* infected human monocytes with ATP we observed a loss in monocyte viability confirming apoptosis with no reduction in bacterial viability. The reason for this discrepancy is under investigation (unpublished data).

Since human monocytes in spite of activation with LPS, IFN γ, TNF α, PMA and ATP did not alter the fate of *M. tuberculosis* in the intracellular environment, attempts were made to study the fate of *M. tuberculosis* inside the peritoneal macrophages from BCG-vaccinated guinea pigs *in vitro*. Peritoneal macrophages from non vaccinated animals served as control. No difference could be observed between the macrophages from vaccinated and non vaccinated animals with respect to the killing of *M. tuberculosis* (unpublished data).

We also infected human monocytes and guinea pig peritoneal macrophages with *M. smegmatis* which is a non pathogen.

Surprisingly neither human monocytes nor guinea pig peritoneal macrophages was able to kill or inhibit the growth of *M. smegmatis in vitro*. More information need to be gained on macrophage-mycobacteria interaction to enable us to develop mycobactericidal assay *in vitro*, which remains a great challenge.

Conclusion

Although macrophage activation is generally considered pivotal to acquiring resistance to tuberculosis, the underlying mechanisms are far from being understood. In mice, reactive nitrogen intermediates have been found to be responsible for macrophage tuberculosis. In humans there has not yet been an unequivocal demonstration of the involvement of reactive nitrogen intermediates in tuberculostasis, nor for any other antimicrobial effector functions of macrophages. Similarly, in the mice γ interferon induces potent tuberculostasis in macrophages but in humans stimulation by additional cytokines/or 1,25-dihydroxy vitamin D3 may be required. It is very clear that human monocytes require the activation signal in the form of cytokines from T lymphocytes. The cytokines that are actually needed to activate and enhance the microbicidal capacity of macrophages, the concentration at which they are required to activate, are not clearly known till date. If these informations are known clearly recombinant cytokines can be given as immunomodulating agents as an adjunct to chemotherapy, thereby making the treatment simpler.

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