PRESENCE OF MYCOBACTERIA IN OTHER CELLS OF LUNG AND LIVER EXEPT IN MACROPHAGES

НАЛИЧИЕ НА МИКОБАКТЕРИИ В ДРУГИ КЛЕТКИ НА БЕЛИЯ И ЧЕРЕН ДРОБ ОСВЕН В МАКРОФАГИТЕ

Dimitrina Kazachka

National Diagnostic and Research Veterinary Medical Institute,
15 "Pencho Slaveikov" Blvd., 1606 Sofia, tel: +359 952 39 03, e-mail: ikazachka@yahoo.com

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ABSTRACT

Macrophages are the key cells for invading and replication of mycobacteria in the host and they play principal role in the pathogenesis of the tuberculosis. The aim of the present study was to reveal if mycobacterium invade other cells except these of immune defense and macrophages first of all as a common feature. The results of ultrastructural studies of lungs of Mycobacterium bovis intraperitonealy infected rabbits and livers of Mycobacterium avium subcutaneously infected chickens showed the presence of mycobacteria into the cytoplasm of pneumocytes II type and capillary endothelial cells of rabbit lungs as well as in the cytoplasm of chicken liver parenchyma cells. On the base of these results and in particular invading of pneumocytes II type as a producers of the surfactant it was suggested that the surfactant or some of his components likely enhance phagocytosis of mycobacteria by macrophages. It could be a reason for mycobacterium affinity to invade pneumocyte II type and to manipulate quality and quantity of the surfactant or some of his components. These results show that M. bovis invaded pneumocytes II type in vivo and it is an important step may be in the investigation of the possibility role of these cells in the pathogenesis of lung infection.

Keywords: Mycobacterium bovis, Mycobacterium avium, macrophage, peumocyte II type, endothelial cell, hepathocyte, rabbits, chicken

РЕЗЮМЕ

Макрофагите са ключови клетки за инвазиране и размножаване на туберкулозните бактерии, които играят основна роля в патогенезата на туберкулозата. Целта на настоящето изследване беше да се установи туберкулозните бактерии инвазират ли и други клетки, освен принадлежащите към имунната защита и главно макрофагите като тяхна обща черта. Резултатите от ултраструктурните изследвания на белите дробове на зайци, заразени интраперитонеално с M. bovis и тези на черния дроб на пилета, заразени подкожно с M. avium показаха присъствие на микобактирии в цитоплазмата на пневмоцити II тип и ендотелните клетки на белодробни кръвоносни капиляри както и в цитоплазмата на чернодробни паренхимни клетки. Въз основа на получените резултати и особено тези, показващи инвазирането на пневмоцити II тип, синтезиращи сърфактанта, може да се предположи, че сърфактанта или някои от негови компоненти, вероятно повишават фагоцитозата на микобактериите от макрофагите. Това би могло да бъде причина за афинитета на микобактериите към инвазиране на пневмоцити II тип и манипулиране качеството и количеството на сърфактанта или неговите компоненти, което да улеснява проникването им в макрофагите. Тези резултати показват, че M. bovis инвазира пневмоцити II тип in vivo и това е важна стъпка в изследванията за вероятността тези клетки да играят съществена роля в патогенезата на белодробните инфекции.

Ключови думи: Микобактериум бовис, Микобактериум авиум, макрофаг, пневмоцит II тип, ендотелна клетка, чернодробна клетка, зайци, пилета

РАЗШИРЕНО РЕЗЮМЕ

В резултат на задълбочените проучвания върху патогенезата на туберкулозата най-многостранно е проучен афинитетът на микобактериите към макрофагите и тяхното взаимодействие. Проникването на туберкулозните бактерии в макрофагите се смята за ключов механизъм, чрез който тези бактерии, прилагайки различни стратегии успяват да овладеят и манипулират вътреклетъчните молекулярни механизми на макрофагите за създаване на благоприятни условия за генната им екпресия и естественото прогресиране на заболяването. Процесите на преодоляване на гостоприемниковата защита и овладяването на вътреклетъчните механизми в инвазираните клетки създават жизненоважен и дълготраен комфорт за туберкулозните бактерии, което прави изключително трудна борбата с предизвиканото от тях заболяване както при хората, така и при животните. Наличието на микобактерии и в други клетки дава основание да се предположи, че туберкулозните бактерии освен, че проявяват тропизъм към макрофагите, те притежават и рецептори, за свързване и инвазиране и на други белодробни клетки. Целта на настоящите изследвания е да се проучи in vivo проникват ли микобактериите и в други нефагоцитиращи клетки както в белите дробове на зайци при директното им интраперитонеално заразяване с M. bovis, така и в черния дроб на пилета, заразени субкутанно с M. avium преди генерализирането на заболяването. Заразяването на зайците беше осъществено с 30-дневна култура на щам M. bovis 123, изолиран от бял дроб на крава, а на пилетата с щам M. avium 7/95, изолиран от лимфен възел на крава. Бактериалната маса за заразяване беше получена чрез посяване на щамовете върху твърда среда на Lowenstein-Jensen с пируват за M. bovis и глицерин за M. avium. Три месеца след заразяването, от евтаназираните животни бяха взети проби от бял и черен дроб и подготвени за наблюдение с електронен микроскоп по стандартната методика за трайно включване в епоксидна смола Дуркупан и контрастиране на ултратънките срези с уранилацетат и оловен цитрат [26]. Резултатите от ултраструктурните изследвания показват, че в пневмоцити II тип и микроваскуларните ендотелни клетки на белите дробове както и в хепатоцитите на черния дроб има наличие на микобактерии. На базата на получените резултати и тези от по-ранните ни изследвания може да се предположи, че една от вероятните причини за афинитета на M. bovis към белите дробове и по-конкретно-пневмоцити II тип е наличието на сърфактант, чието синтезиране протича именно в тях. Възможността за манипулиране синтезирането на сърфактанта или неговите компоненти, които служат за маскиране и съответно повишаване фагоцитозата на туберкулозните бактерии от макрофагите, като естествени "чистачи" и на отработения сърфактант, вероятно играе съществена роля и в патогенезата на туберкулозата. Въз основа на наблюдаваната морфологична картина на наличие на M. avium в чернодробните паренхимни клетки и данните за освобождаване и пренасяне чрез екзозоми на специфични компоненти от повърхността на микобактериите в инфектираните клетки би могло да се допусне, че намиращите се в Купферовите клетки екзозоми биха могли да преминат през фенестрите на ендотелните клетки на синусите и да проникнат в хепатоцитите, повлиявайки молекулярните клетъчни процеси за последващо инвазиране от микобактериите.

INTRODUCTION

As it is well known, the mycobacterium is among the very dangerous microorganisms and most successful at adapting to long-term residence in the host of all time. Many species of environmental mycobacteria infect animals and have zoonotic potential which is very important to the economy and research [15]. Aerosol transmission is the predominant route of infection whereby droplet nuclei containing one organism is enough to establish an infection. The most often this infection begins in the respiratory system and the main site of mycobacteria -more than 80% is in the lung [20, 21, 22]. The ability to infect macrophages is a common characteristic shared of mycobacterium species for surviving and replication. According to McDonough et al. [20] a central to understanding the pathogenesis of tuberculosis is the interaction between the pathogen and macrophages.

Through a variety of cell surface receptors,

macrophages internalize mycobacteria into phagosomes that undergo maturational events and expose the microbes to acid, lytic enzymes, oxygenated lipids, fatty acids, reactive oxygen and nitrogen intermediates [1, 23]. In some cases mycobacteria use alternative pathways to be taken by macrophages including surfactant protein receptors in the lung, scavenger receptors and some molecules [8, 11, 13, 14, 16, 17]. According to Bermudez et al. [6] this factors may represent manner of adaptation to diverse environments and a specific site or ability to enter mononuclear phagocytes in different stages of maturation. At the same time successful intracellular pathogens have developed different strategies before macrophages to be immunological activated allowing them to survive within phagosomes including: arresting the maturation of the early endosome to a phagolysosome by inhibiting fusion of the mycobacterium-containing phagosome with lysosomes, resistance against antimicrobial molecules, and adaptation to host-induced metabolic constraints [2, 12, 15]. Pathogenic mycobacteria modulate macrophage signaling responses and thereby limit the ability of macrophages to produce or respond to immune modulators. In this way the infected cells become progressively unresponsive to further activation by cytokines and the pathogen undergoes intracellular replication [10, 18, 29]. Moreira et al. [23] observed that in dividing of mycobacteria the vacuole appears to divide with them, sequestering each bacillus in separate tight vacuole. According Gomes et al. [15] the ability of mycobacteria to remain within a tight vacuole may facilitate their control of phagosome fusion with other vacuoles within the cells. According Bhatnagar and Schorey [7] the capacity of mycobacterium to alter macrophage functions requires the expression of specific mycobacterial surface components. Different species mycobacteria use one or more surface components in the process of pathogenesis. Some of them can to be repeated in the species, but probably one is predominant for altering host microbicidal function or in inducing a proinflammatory cascade as this is on the cell surface of the M. tuberculosis, M. avium and M. bovis. There is also evidence showing that these lipids of mycobacteria cell wall accumulate inside infected cells and could be trafficked to other cellular compartments and modulate cellular functions [19, 27]. Bhatnagar and Schorey [7] and Beatty et al. [3] demonstrated that mycobacterial surface cell wall lipids are released in infected macrophages and trafficked to a distinct compartment by endocytic pathway designated the multivescular body /MVB/. Earlier studies of Raposo et al. [25] have shown that fusion of MBVs with the plasma membrane results in the release of small 50-100 nm vesicles called exosomes. Bhatnagar and Schorey [7] found that exosomes released from M. avium-infected macrophages can interact with uninfected macrophage leading to their retention in these "bystander" cells. These authors for the first time showed that the exosomes carrying bacterial components isolated from mycobacteria-infected macrophage can induce a proinflammatory response and suggested a novel mechanism by which stimulator molecules present on intracellular pathogens can be released from infected cells to promote an immune response.

Although mycobacteria evolved to survive inside phagocyte cells, they can enter and replicate in other host cells as in pneumocytes of the lung [5]. Mehta et al. [20] observed entry and intracellular replication of M. tuberculosis in cultured human microvascular endothelial cells. Mills et al. [22] proved that M. tuberukulosis is capable to infect liver parenchyma cells in immune deficient mice. The activation of some host cell proteins may specifically recruit other proteins to the vacuole membrane, creating the conditions for the uptake of needed nutrients for bacterial replication, virulence gene regulation and expression associated with the natural progression of the disease [4, 9, 28].

As it is visible, an abundance of dates there are for the pathogenesis of tuberculosis but most of these dates are connected with interaction of bacilli with cells of host defense system and first of all with macrophages. The information about invading of other host cells by mycobacteria is relatively smaller. The aim of the present study was to investigate the possibility present of M. bovis and M. avium in other cells then macrophages in rabbits and chickens.

MATERIALS AND METHODS

For the infection of rabbits was used 30-ty day

culture of M. bovis species 123 isolated from the cow lung and for chickens - M. avium 7/95 isolated from the cow lymph nodule. The species were grown on Lowenstein-Jensen solid medium with piruvate for M. bovis and glycerin for M. avium at 37^0 C. The density of bacterial masse was $1X10^6$ CFU. Studies were done on eight rabbits infected intraperitonealy with dose 0, 5 mg/kg M. bovis and eight chickens infected subcutaneously in a dose 1ml per M. avium. Three months after infection they were immobilize by 2% ksilasin consequence by euthanasia with euthanasin injected intracardialy.

The etiology of infection in different organs was proved by PSR of species DNK [3].

For electron microscopy the pieces of lungs and livers were fixed for 2 h in 4% glutaraldehyde solution at 4^0 C, then washed in cacodilate buffer and post fixed for 1h in 1% osmium tetroxide at 4^0 C in refrigerator. After dehydration stepwise in graded series of alcohol the pieces were embedded in Durcupan [26]. The ultra thin sections were double stained by uranyl acetate and lead citrate for examination with Tesla BS 500 transmission electron microscope.

RESULTS AND DISCUSSION

As it is known the macrophages are the most convenient cells for survive and replication of pathogenic mycobacteria species. The mechanism of mycobacterium interaction with professional phagocytes most of all macrophages has been extensively studied during the past years [1, 2, 7, 8, 18]. The present of mycobacteria into the lung macrophages were observed also in our electron microscopy studies of intraperitonealy infected with Myicobacterium bovis rabbits /fig. 1/.

Although the ability to infect macrophages is a common characteristic shared among mycobacterial species, our ultrastructural studies of the lung showed M. bovis not only into macrophage phagosomes but also into cytoplasm vacuoles of pneumocyte II type /fig. 2/. Our results confirmed in vivo the first step toward investigation the possibility of mycobacteria to infect pneumocytes II type examined in vitro by Bermudez and Goodman [5]. They have suggested that M. tuberculosis invasion and replication into II type alveolar cells is associated with pathogenesis of this disease. In support of this hypothesis are the studies in vivo of Gaynor et al. also [13]. According them the surfactant protein A /SP-A/ produced by pneumocyte II type mediates enhanced phagocytosis of M. tuberculosis by a direct interaction with macrophages. On the base of this results it comes naturally to tolerate that mycobacteria would be try to find a way for mastering and controlling of surfactant or some of his components. We suggest that after fallen of mycobacteria into distal airspace the present macrophages ingest some of them but others tubercle bacilli use the available exocytosed surfactant or some of his proteins as a mask and invade pneumocyte II type. It is probable because

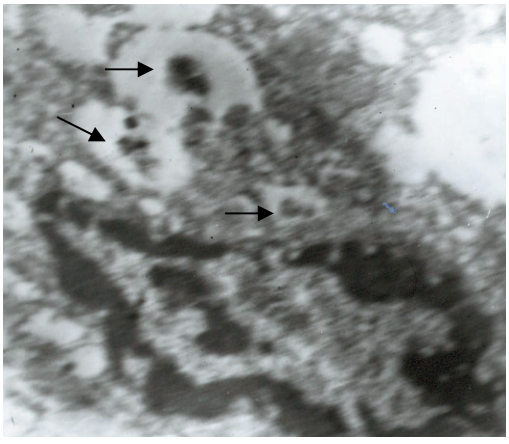

Fig.1 M. bovis into fagosomes of rabbit lung macrophage

Фиг.1 M. bovis във фагозоми на макрофаг в заешки бял дроб

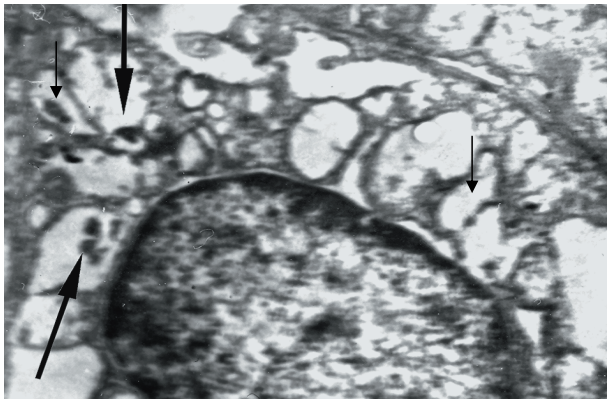

Fig. 2 Pneumocyte II type. M. bovis are into vacuoles formed in the place of exocytosed lamellar bodies

Фиг. 2 Пневмоцити II тип. Наличие на M. bovis във вакуоли, формирани на мястото на екзоцитирани ламеларни телца

the pneumocytes II type are the place not only for producing but end for recycling of part work off surfactant.

In pneumocyte II type mycobacteria were disposed into vacuole occupying the place of exocytosed lamellar bodies, possible as an appropriate site not only for surviving and a convenient niche for replication but and for manipulation of the surfactant production or his different components.

At the same time these pneumocytes II type present antigen themselves or through dendritic cells to the circulating T-cells and to the macrophages directly or to the both. It is possible because according to Cunningham et al /1994/ pneumocyte II type possess major histocompatibility complex class II molecules and co-stimulatory signals to intracellular adhesion molecule-1 and B7 needed for effective antigen presentation on their outer membrane. Receiving antigen information directly or indirectly macrophages activate and rapid influx into respiratory surface. In this way mycobacterium could be use the pneumocyte II type and surfactant for: 1/ surviving and replication; 2/ manipulation of surfactant production; 3/ attraction of macrophages; 4/ enhancement of their phagocytosis. This manner the mycobacteria facilitate own successfully invasion of macrophages in consequence which are normal cleaners of work off surfactant. In support of our opinion were observed mycobacteria situated in the center or among lamellas of surfactant lamellar bodies engulfed by macrophage /unpublished studies/. We support opinion of Bermudez and Goodman [5] that pneumocytes II type play an important role in the pathogenesis of lung tuberculosis. It is possible that the reason for the affinity of mycobcteria to these lung cells is the production of surfactant mostly. The conclusion of Bermudes et al. [6] that particular reason why mycobacteria are able to bind and invade cells via so many receptors is unknown and suggested that this may represent different strategies, adapted to diverse intracellular environments, the availability of specific nutrients, and the protection against host defense mechanisms.

By electron microscope analysis of the same infected rabbit's lung we established the presence an abundance of mycobacteria in alveolar lumen. At the same time mycobacteria there were into vacuoles of endothelial cells of lung capillary also /fig.3/. The mycobacteria were along or a few in vacuoles of endothelial cytoplasm. Some of mycobacteria were seen close to the alveolar lumen cell membrane in endothelial cells. It was a reason to thing that bacilli enter into the cytoplasm of endothelial cells through invagination of cell membrane. It is possible the endothelial cells to play a role of door for incoming or leaving of blood stream by mycobacteria in different places and generalization of disease. Our findings of mycobacteria in endothelial cells as potential host cells during lung infection confirm results of the experiments in vitro of Mehta et al. [21].

Ultrastructural investigations of liver from the chicken infected subcutaneously with M. avium surprised us by the presence of mycobacterium into hepatocyte vacuole in contrast to the opinion of some researcher's [28]. In the hepatocyte cytoplasm there were many different in size vacuoles with bacteria. In some of them the mycobacterium was along, but in others they were a few.

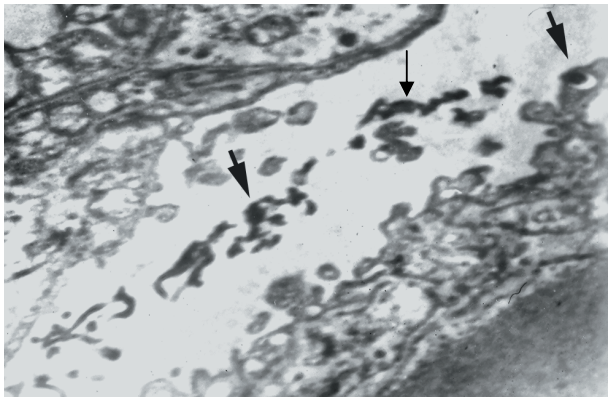

Fig. 3 M. bovis is visible into vacuoles of rabbit lung capillary endothelial cell. Abundance of mycobacteria there are in alveolar lumen.

Фиг. 3. M. bovis се наблюдава във вакуоли на ендотелни клетки от заешки бял дроб Наличие на множество микобактерии в алвеоларния лумен

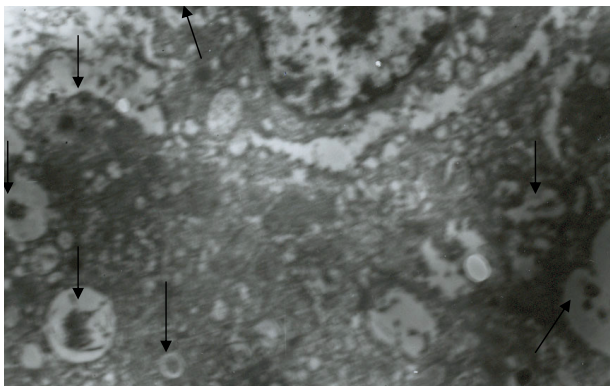

Fig. 4 M. avium is disposed into vacuoles of hepatocyte. In cytoplasm vacuole of Kupffer cell there is mycobacterium

Фиг. 4 Наличие на M. avium в цитоплазмени вакуоли на хепатоцит. Микобактерии в цитоплазмена вануола на Купферова клетка

At the same time Kupffer cell with mycobacteria was in tight contact with a part of sinusoid endothelial cell and hepatocyte, as well as was visible endocytose of mycobacteria into hepatocyte also /fig. 4/.

According to Seiler et al. [28] liver parenchyma cells do not have the opportunity to phagocytose of mycobacteria in vivo due to the competition with liver macrophages, which pick up bacteria immediately and control infection, or due to the special microanatomy of the liver. Their opinion is that the liver sinusoid lined by endothelial cells, allowing the exchange of small molecules between the sinusoid and the liver parenchyma cells and may be physically prevented access of mycobacteria to liver parenchyma cells. Their experiments indicate that, even in the absence of macrophages, liver parenchyma cells do not have the opportunity to pick up mycobacteria in vivo. Sinusoidal endothelial cells shield parenchyma cells and may prevent initial contact between mycobacteria and liver parenchyma cells in vivo. But Mills et al. [22] suggested that virulent M. tuberculosis and M. bovis are capable to infect parenchyma cells. There is also evidence showing that the lipids of mycobacteria cell wall accumulate inside infected cells and could be trafficked to other cellular compartments and modulate cellular functions [18]. Bhatnagar and Schorey /7/ hypothesized that mycobacterial cell wall lipids are released from the mycobacteria surface in infected macrophages and trafficked to a distinct compartment within the endocytic pathway designated the multivesicular body /MVB/. Earlier studies of Raposo et al. [25] have shown that fusion of MBVs with the plasma membrane results in the release of small 50-100 nm vesicles called exosomes which can interact with uninfected macrophages. These authors for the first time showed that the exosomes carrying bacterial components could induce a proinflammatory response and suggested a novel mechanism by which stimulator molecules present on intracellular pathogens can be released from infected cells.

On the base of all these dates and our results for different mycobacteria strategies to modulate cellular function for surviving and replication we suggested that it is possible these mechanisms to take carry for function of other cells than macrophages. It is possible also that the mechanisms of mycobacteria to invade different cells to be more complex and need by some other unknown factors. In the future it will be interesting to clarify more about how and why the mycobacteria invade different of macrofages cells. In conclusion it would to say that one of the reason mycobacteria to invade different from the phagocytosing cells as a pneumocyte II type is an very important part of pathogenesis of tuberculosis disease probably and needs by further investigations.

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