Live antigen carriers as tools for improved anti-tuberculosis vaccines

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Abstract

Recombinant (r) Mycobacterium bovis BCG strains have been constructed which secrete biologically active listeriolysin (Hly) fusion protein of Listeria monocytogenes. In human and murine macrophage-like cell lines, intracellular persistence of these r-BCG strains was reduced as compared to the parental BCG strain. By immunogold labelling Hly was detected in membrane structures and within the phagosomal space of macrophages. Hly fusions consistently co-localized with a lysosome-associated membrane glycoprotein (LAMP-1) suggesting that membrane attack conformation of Hly was not altered. Although r-BCG microorganisms apparently did not egress into the cytoplasmic compartment of host cells, they both improved major histocompatibility complex class I presentation of co-phagocytosed soluble ovalbumin as compared with wild-type BCG microbes. These data suggest that Hly secretion endows BCG with an improved capacity to stimulate CD8 T cells. Because CD8 T cells play a major role in protection against tuberculosis such Hly-secreting r-BCG constructs are anti-tuberculosis vaccine candidates. In addition, we report on our r-Salmonella typhimurium expression system combined with the HlyB/HlyD/TolC export machinery for delivering the prominent mycobacterial antigen Ag85B for immune recognition. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Since the turn of this century when tuberculosis (TB) was the number one killer amongst all infectious agents in Western Europe and the United States, mortality incidences have drastically declined in these regions [1]. Yet, TB caused by Mycobacterium tuberculosis remains a significant health problem globally. It is estimated that one-third of the world population is infected with M. tuberculosis. Worldwide, 30 million people suffer from active TB, whilst annually 3 million new cases arise and 8 million people die of TB [1]. In several Eastern European states, including Russia and Romania, incidences of TB have risen dramatically during the last decade. Accordingly, there is an urgent need for more efficacious anti-TB vaccines that could prevent TB or even cure active disease.

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2. Immunological implications of TB for vaccine development

Fewer than 10% of the two billion people that harbor *M. tuberculosis* will eventually suffer from clinical TB [2]. It may be surprising that *M. tuberculosis*, the pathogen that kills more lives than any other infectious agent, does little harm to most individuals it has infected. Generally, TB unfolds through reactivation of dormant *M. tuberculosis* microorganisms which are well controlled by a competent immune system. Immunodeficient individuals have a highly increased risk of developing TB shortly after *M. tuberculosis* infection. Therefore, it is not surprising that human immunodeficiency virus infection impairing central effector functions of the immune system performed by CD4 T cells does little harm to most individuals it has infected. Generally, TB unfolds through reactivation of dormant *M. tuberculosis* microorganisms which are well controlled by a competent immune system. Immunodeficient individuals have a highly increased risk of developing TB shortly after *M. tuberculosis* infection. Therefore, it is not surprising that human immunodeficiency virus infection impairing central effector functions of the immune system performed by CD4 T cells does little harm to most individuals it has infected. Generally, TB unfolds through reactivation of dormant *M. tuberculosis* microorganisms which are well controlled by a competent immune system. Immunodeficient individuals have a highly increased risk of developing TB shortly after *M. tuberculosis* infection. Therefore, it is not surprising that human immunodeficiency virus infection impairing central effector functions of the immune system performed by CD4 T cells does little harm to most individuals it has infected.

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Because *M. tuberculosis* resides in a large proportion of the world population in a clinically unapparent form, two types of anti-TB vaccines are probably required, namely a preventive vaccine, targeted at the noninfected population, and a therapeutic vaccine, targeted at the already infected, still healthy population. It remains to be established whether a single vaccine can satisfy both goals.

3. Importance of CD8 T cells for TB prevention and therapy

*M. tuberculosis* belongs to the group of intracellular bacteria, like *Salmonella typhimurium*, which replicate within the phagosomal vacuoles of resting macrophages [2]. Most intracellular microorganisms including *M. tuberculosis* and *S. typhimurium* impair phagosome maturation and thus grow in an altered vacuolar compartment [3,4]. It is generally assumed that antigens from pathogens remaining in the phagosome are primarily presented by major histocompatibility complex (MHC) class II molecules to CD4 T cells [5]. In contrast, bacterial antigens rec-
ognized by CD8 T cells via MHC class I gene products originate from bacteria, like *Listeria monocytogenes*, which replicate in the cytoplasm of macrophages [2]. The central role of CD4 T lymphocytes in TB control is beyond question. These CD4 T cells are of the Th1 type, i.e. they are potent producers of interferon-γ (IFN-γ) which, in turn, activates antimycobacterial activities in macrophages [5]. Yet, it is likely that this T cell population alone does not suffice for full protection. Several studies in experimental animal models and humans have shown that *M. tuberculosis* microbes not only stimulate antigen-specific, MHC class II-restricted CD4 T helper cells [5] or cytotoxic CD4 T lymphocytes [6,7] but also CD8 cytotoxic T cells (CTL) [8]. Aside from CD8 CTL which are stimulated by and perform their effector functions via antigen recognition controlled by classical MHC class Ia alleles [9], more recently additional ‘unconventional’ CD8 T cells have been identified: (i) a T cell subset which recognizes mycobacterial mycolic acid or lipoarabinomannan via CD1a, CD1b or CD1c molecules [10]; (ii) a group of CD8 T cells that identifies antigens on non-polymorphic MHC class Ib receptors [11].

In mice the important role of CD8 T cells was convincingly demonstrated by the failure of β2-microglobulin (β2m)-deficient mice to control experimental *M. tuberculosis* infection [12]. Because these
β2m-mutant mice lack MHC class Ia, MHC class Ib, and CD1 molecules, functional CD8 T cells cannot develop. Mice in which the gene for the CD8 molecule has been disrupted are also highly susceptible to *M. tuberculosis* infection [13]. It was additionally reported that β2m-deficient mice are capable of controlling low inocula of the BCG strain, the current anti-TB vaccine [12,14].

This differential CD8 T cell dependence between *M. tuberculosis* and BCG may reflect the availability of mycobacterial antigens for MHC class I presentation in antigen-presenting cells (APC). *M. tuberculosis*-specific antigens could have better access to the host cell cytoplasm than antigens from BCG leading to more pronounced MHC class I presentation. Consequently a more effective CD8 T cell response is generated by *M. tuberculosis*. This notion was recently supported by in vitro experiments showing increased MHC class I presentation of a bystander antigen, soluble ovalbumin (OVA), by simultaneous *M. tuberculosis*, but not BCG, infection of APC [15].

4. BCG vaccination

Already in 1908, the BCG vaccine had been developed by attenuation of *Mycobacterium bovis* through more than 230 passages on bile glycerol agar [16]. Since 1948, BCG has been used to vaccinate 3 billion people against TB, with negligible side effects. It can be administered intradermally as a single inoculum at any time after birth, and its efficacy is unaffected by maternal antibodies. Successful BCG vaccination leads to minor lesions, local self-limiting bacterial multiplication and development of delayed-type hypersensitivity (DTH) against mycobacterial proteins which may persist for 5–50 years. Noteworthy, in the absence of BCG vaccination this DTH response is considered a tentative indicator of active *M. tuberculosis* infection, and therefore BCG is not recommended for TB control in countries with low TB incidences.

Although BCG is the most widely used vaccine worldwide, its application is discussed controversially. General agreement exists that BCG can protect or at least ameliorate severe forms of systemic TB in children, particularly meningitis [17]. Yet, it seems to be of low or no protective value in adults.

The most prevalent form of TB, namely reactivation of dormant TB in adults, cannot be prevented by this vaccine in a satisfactory way [17]. In various controlled trials, the protective efficacy of BCG vaccination ranged from ineffectiveness to 80% protection [18]. Several reasons may be responsible for this variation in protection including: (i) genetic variability and different age of the vaccinated individuals; (ii) unique characteristics of environmental mycobacterial species prevalent in different parts of the world; (iii) the use of different BCG strains [BCG Copenhagen (Danish 1331) represented the standard vaccine of the World Health Organization until 1970 only]; (iv) variable doses of BCG used for immunization; (v) different immunization schedules.

Although the disease can be treated successfully, provided it is not caused by multidrug-resistant (MDR) strains, general agreement also exists that
TB will not be eradicated from the world by chemotherapeutic regimes. Unfortunately, *M. tuberculosis* is no exception within the microbial world and hence possesses the capacity to become resistant to various anti-tuberculosis drugs [19,20]. In fact, increased incidences of MDR strains of *M. tuberculosis* have
evoked reconsiderations about the need for vaccines to control TB. Many explanations for the failure of BCG have been brought forward, amongst which two are based on immunological deficits:

1. BCG lacks important ('protective') antigens. Indeed, at least two gene clusters (RD1, RD2) in the genome of virulent M. bovis have been identified which are absent from BCG and present in clinical isolates of M. tuberculosis [21,22].

2. BCG fails to stimulate the combination of T lymphocytes required for protection against TB [23]. As discussed above, TB control may depend on CD4 and CD8 T cells as well as in humans. In contrast, BCG is primarily a stimulator of CD4 T cells and thus fails to stimulate optimum protection efficiently.

Obviously, any future anti-TB vaccine should not only mimic the advantages of BCG, in particular low side effects and potent stimulation of CD4 T cells. It must also overcome the pitfalls of BCG. One way to circumvent these obstacles is to improve BCG itself. First, it is possible to reconstitute BCG with M. tuberculosis-specific genes of the RD1 or RD2 region. Care must be taken to avoid re-establishment of virulence by bringing back missing virulence genes. Recombinant (r) BCG expressing pore-forming cytolysins could be engineered which could overcome the strict compartmentalization of antigens delivered by BCG. The two ways of improving BCG are not mutually exclusive and both prototypes could be combined to yield r-BCG which express M. tuberculosis-specific antigens and are capable of egressing into the cytosol. The improved BCG would also profit from the advantage of possessing mycobacterial glycolipids required for stimulation of CD1-restricted T cells.

5. BCG expressing pore-forming cytolysins

The phagosomal escape function of biologically active listeriolysin (Hly) of L. monocytogenes represents a unique mechanism to facilitate MHC class I antigen presentation of listerial or bystander antigens [24]. Hly, a pore-forming sulfhydryl-activated cytolysin, is essential for the release of the complete L. monocytogenes microorganisms from phagosomal vacuoles into the cytoplasm of host cells [25,26]. Recently, this escape function was successfully transferred to Bacillus subtilis and to attenuated Salmonella dublin or S. typhimurium strains [27,28]. As a corollary, two r-BCG strains pMV306:Hly and pAT261:Hly secreting hemolytically active Hly were constructed with direct Hly expression via chromosomal integration of the mycobacterial shuttle vector or by episomal plasmid replication, respectively [29]. Both hemolytic r-BCG strains showed a lower persistence in the murine macrophage like-cell line J774A.1. At day 15 post infection (p.i.) viable bacteria of BCG pMV306:Hly strain were not detected in infected macrophages, suggesting sterile elimination of this construct at least in the presence of gentamicin which kills extracellular r-BCG bacteria [29].

This feature, in turn, may be explained by an increased cytolytic effect of r-BCG microorganisms secreting Hly within host cells in long-term culture. In contrast, cytolytic effects of hemolytic BCG strains as measured by lactate dehydrogenase (LDH) release into the supernatant fluid of infected macrophages were not detected in short-term culture experiments [29]. Virulent L. monocytogenes capable of leaving the phagosomal compartment between 2 h and 4 h p.i., however, caused LDH release of infected host cells at 24 h p.i. Therefore, it may not be surprising that r-BCG secreting Hly were still observed inside their phagosomal niche of macrophages at this time point (Fig. 1). Immunoelectron microscopical analysis of macrophages infected with BCG pAT261:Hly or BCG pMV306:Hly revealed a similar intraphagosomal distribution of bacilli as compared to wild-type BCG at 24 h p.i. [29]. Labelling with affinity-purified rabbit anti-Hly antibody demonstrated synthesis and release of Hly from the intracellular bacteria, although the mycobacterial constructs apparently remained within their vacuoles. The Hly signal was not only observed in bacilli and the surrounding lumenal space, but also in small vesicles distinct from the bacteria containing vacuoles, and consistently Hly was associated with the LAMP-1 indicative for late endosomal lysosomes (Fig. 1) [29].

This integration of Hly into lysosomal membrane structures of vacuoles harboring r-BCG together
with the hemolytic activity – at acidic pH – of supernatant fluids harvested from r-BCG cultures suggests that membrane pores were generated which facilitated access of mycobacterial proteins to classical MHC class I presentation pathways (Fig. 2). Instead of mycobacterial antigens we used OVA, as defined target in order to determine MHC class I antigen delivery by r-BCG constructs. Macrophage-like cells were simultaneously incubated with 50 µg ml⁻¹ OVA and BCG pAT261:Hly or BCG pMV306:Hly or parental BCG microorganisms at different multiplicities of infection (moi).

BCG pAT261:Hly and BCG pMV306:Hly microorganisms facilitated access of OVA to the MHC class I presentation pathway in IFN-γ-activated and mycobacteria-infected macrophages as indicated by MHC class I-specific OVA (Fig. 2). Instead of mycobacterial antigens we used OVA, as defined target in order to determine MHC class I antigen delivery by r-BCG constructs. Macrophage-like cells were simultaneously incubated with 50 µg ml⁻¹ OVA and BCG pAT261:Hly or BCG pMV306:Hly or parental BCG microorganisms at different multiplicities of infection (moi).

Antigen delivery of a single mycobacterial protein by S. typhimurium aroA

Provided that protective immunity against TB can be induced by a few or even a single antigen, heterologous bacterial or viral vectors expressing M. tuberculosis-specific antigens or subunit vaccines composed of defined M. tuberculosis antigens and an appropriate adjuvant may be considered as vaccine candidates against TB. Attenuated r-S. typhimurium aroA strains have been most widely used as carriers of heterologous antigens from various pathogens [30,31]. The recently described S. typhimurium aroA strains capable of secreting heterologous antigens appear promising candidates in this respect [32–34]. This Salmonella-specific antigen delivery system expressing listerial Hly or p60 antigens induced both CD4 and CD8 T cells which could adoptively confer protective immunity against L. monocytogenes infection in mice which is primarily controlled by CD8 T cells [32]. A fusion protein was constructed encompassing Ag85B of BCG and the C-terminal part of hemolysin (HlyA) of uropathogenic and hemolytic Escherichia coli isolates, respectively. The latter fusion partner directs protein secretion via recognition of the HlyB/HlyD/TolC translocator across the cell wall of Gram-negative E. coli or Salmonella microorganisms (Fig. 4). The antigen Ag85B of BCG and M. tuberculosis belongs to a family of proteins with fibronectin-binding capacity and mycolyltransferase activity which is involved in the final stages of mycobacterial cell wall assembly [35]. Noteworthy, Ag85B of BCG shows 99% homology to its cognate of M. tuberculosis. Besides these biochemical features, Ag85B represents a well-studied immunodominant antigen for humoral and cell-mediated immunity in mice and humans [36]. Moreover, antigen Ag85B is a secreted protein and hence an early target of the T cell response against M. tuberculosis [37].

The r-S. typhimurium Ag85B strain secretes the Ag85B-HlyA fusion protein into the lumen of vacuoles within infected macrophages. This antigenic fusion protein appears to localize within intracellular compartments similar to the Ag85B proteins secreted by BCG. The difference in Ag85B delivery between r-Salmonella and BCG lies in the increased occurrence of vesicles containing only bacterial antigens, but lacking whole microorganisms in the case of BCG as compared to S. typhimurium infection of macrophages ([29,38], Hess et al., unpublished results).

This phenomenon probably reflects differential efficiency of antigen processing by the two bacterial microbes. Together with the potent CD4 Th1 cell induction by Salmonella carrier strains after oral administration, this feature may be advantageous for vaccine development against TB. Currently protec-
tion experiments with *S. typhimurium* secreting Ag85B vaccine constructs are being performed in experimental animal models.

7. Concluding remarks

The present paper describes our attempts towards the development of an effective vaccine against TB. Currently, two major avenues are being pursued: first, identification of protective antigens (i.e. proteins that are absent from BCG, present in *M. tuberculosis* and immunodominant for protective T cells); second, construction of improved BCG vaccine strains which stimulate the optimum combination of T cell populations required for protection. It is not yet known which approach will succeed and it is quite possible that a combination of both (i.e. expression of protective antigens by improved BCG) will provide the most efficient measure. Our efforts have focused on improving BCG by endowing it with the capacity to introduce antigens into the MHC class I pathway in a more efficient way. However, we also consider identification of additional protective antigens as important. Thus far, we have restricted ourselves to well known proteins such as antigen Ag85B expressed by potent viable vaccine carriers. With the *M. tuberculosis* genome being available, identification of novel proteins with potential value will be facilitated. Future experiments will show whether a broad array of T cells is required for protection or, alternatively, whether a limited number of antigen-specific T cell clones are sufficient. In the first case, improved BCG constructs expressing additional *M. tuberculosis*-specific antigens would be the strategy of choice. In the latter case, subunit vaccines or heterologous r-vaccine carriers expressing a limited number of immunodominant protective antigens would be satisfactory.

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