Prospects for Designing New Generation Anti-Tuberculosis Vaccines¹

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Abstract—Recent progress in designing new vaccines against tuberculosis is concisely reviewed. Data on administration of various secreted *Mycobacterium tuberculosis* antigens as components of subunit vaccines are briefed. The need in constructing artificial antigens comprising several *M. tuberculosis* antigens (epitopes) is illustrated by particular examples. The prospect of developing an “edible” vaccine against tuberculosis involving transgenic plants is discussed.

Keywords: tuberculosis, vaccine, antigens, subunit vaccine, transgenic plants, epitope, edible vaccine.

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INTRODUCTION

The recently established situation with tuberculosis in both Russia and many other countries is catastrophic; moreover, this is the infectious disease with the highest mortality rate. According to the WHO (World Health Organization) data, the annual tuberculosis morbidity is about eight million people with the annual mortality of three million people. A steady increase in the overall mortality rate observed all over the world starting from the early 1990s is associated with HIV spreading, since tuberculosis is the main cause of death of the acquired immunodeficiency syndrome cases: the survival time when these pathologies are combined is one the average 2 months (Ormerod et al., 1994).

A serious problem in treating tuberculosis is emergence of drug resistant *Mycobacterium tuberculosis* strains. The resistant tuberculosis forms caused by these strains have considerably spread in many countries of Asian and African continents.

According to the number and range of the preparations to which *M. tuberculosis* displays resistance, the following variants are considered: multiple drug resistance (MDR), extensive drug resistance (XDR), and total drug resistance (TDR). The MDR strains are resistant to at least two antibiotics, rifampin and isoniazide, the most efficient first-line anti-tuberculosis drugs. The XDR strains are additionally resistant to any of the fluoroquinolones and one or several injection drugs (capreomycin, kanamycin, and amikacin). The TDR strains are resistant to any of the currently known anti-tuberculosis drugs.

It is evident that spreading of drug resistant strains requires both designing new drugs and intensifying the efforts for creation of fundamentally new immunopre-

vention tools. Since 2006, the WHO announced the global program of tuberculosis control—*The Global Plan to Stop TB*—including development of the new generation vaccines as an important component.

TUBERCULOSIS: BRIEF INFORMATION

Tuberculosis is a human and animal disease caused by several mycobacterium species. The genus *Mycobacterium* comprises the aerobic non-spore-forming non-motile bacilli with high lipid content in the cell wall, widespread in environment. Over 40 mycobacterium species are known; however, only few of them are able cause a disease. These species of closely related slow growing mycobacteria are referred to as the *Mycobacterium tuberculosis* complex (MTC) and comprise *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. pinipedi*, *M. caprae*, *M. bovis*, *M. microti*, and *M. avium*. *M. tuberculosis*, discovered by R. Koch in 1882, plays the key role in pathogenesis of the human tuberculosis; however, the human tuberculosis cases in the African regions adjacent to Sahara are caused by the infection with *M. africanum* and *M. canetti*. *M. bovis* causes disease in a wide range of warm-blooded organisms, including humans; *M. microti* is pathogenic for mice and *M. avium*, for birds. In the current microbiological classification, *M. avium* is ascribed to non-tuberculosis *Mycobacterium avium*-intracellulare complex, which causes less widespread human and animal diseases, mycobacterioses.

The bacteria belonging to the tuberculosis complex are genotypically very similar. The homology level of their DNA is 99.9%; however, several phenotypic differences and, especially, the host range allowed the species names to be preserved.

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The main reservoir and source of air-borne tuberculosis infection is a bacillary patient, excreting numerous mycobacteria with sputum and saliva. In addition, infection can be transmitted via an alimentary pathway—by consuming milk and sour milk products of the cows affected with *M. bovis* or eggs of the hens infected with *M. avium*. A contact disease transmission via damaged skin, for example, when milking ill animals, is also possible. Upon an air-borne infection, tuberculosis most frequently develops in the respiratory organs; an alimentary infection pathway can result in affection of the kidneys, lungs, bones and joints, peripheral lymph nodes, urogenital organs, eyes, and central nervous system. Depending on the main clinical manifestations, the pulmonary and non-pulmonary tuberculosis variants are considered. The pulmonary tuberculosis still remains the most widespread and dangerous form of the disease. In this case, any organ, including the skin, can be infected. The most abundant among the non-pulmonary forms are urogenital tuberculosis as well as the tuberculosis of bones and joints and peripheral lymph nodes.

However, infection of humans or animals with mycobacteria of the tuberculosis complex is not a sufficient condition for disease progress, since 90% of the people infected with *M. tuberculosis* never develop an active tuberculosis form (Nagelkerke, et al., 2006). It is assumed that the disease progresses due to certain sometimes temporary and even transient abnormalities in the immune system performance. Several possible mechanisms causing such abnormalities are discussed in the literature, including recently formulated hypothesis on involvement of concurrent viral infections, first and foremost, HIV, in development of the immunity dysfunctions (Hussain, et al., 2007).

IMPROVEMENT OF TUBERCULOSIS VACCINE

Currently tuberculosis prevention involves immunization of newborns with the live BCG vaccine. BCG is a live attenuated *M. bovis* BCG (Bacillus Calmette-Guerin), which preserved its antigenic and immunogenic properties. Manufacture of this vaccine is inexpensive and its administration is safe, which have provided a wide use of the vaccination as the main tool in preventing tuberculosis (Orme, 1999). Since 1948 to 1986, over 2.5 billion persons were vaccinated with BCG; by 1995, the number of vaccinees was about three billion (Jacobs, et al., 1987; Hanson, et al., 1995). The experience in BCG vaccination has demonstrated its high efficiency against tuberculosis development in children and a weak protective activity or even a complete absence of any protective effect against the pulmonary tuberculosis in adults (Jacobs, et al., 1987; Haile and Kallenius, 2005; Kallenius, et al., 2007). The BCG vaccination in children effectively prevents from miliary tuberculosis and tubercular meningitis (Surekha Rani et al., 2005). However, certain doubts about its univer-

sality and efficiency have recently become more pronounced (Mustafa, 2002).

The recorded variation in the BCG protective activity can result from differences in both the immune status of vaccinees and vaccine strains (Maes, et al., 1996; Collins and Kaufmann, 2001). A completed 60-year-long study of the vaccination results in American Indians has demonstrated that a long-term BCG efficiency amounted to 52% (Haile and Kallenius, 2005). Note that similar to other live vaccines, BCG can cause negative side effects (Klein, 2000). In particular, the complications in BCG vaccination are observed in the children prenatally infected with HIV (Hesseling et al., 2004). A frequent complication is inflammation of axillary lymph nodes at the side of injection; in this case BCG is detectable by plating in both the affected lymph node and gastric juice; and these bacteria can be already resistant to some anti-tuberculosis drugs, for example, isoniazide. When treating these side effects of BCG vaccination, BCG can also acquire rifampin resistance, which is determined, as had been demonstrated, by a mutation in the *rpoB* gene. Thus, it is necessary to control the drug resistance of the vaccine both before administration and when treating the vaccination side effects (Hesseling, et al., 2004).

It is now topical to design new generation vaccines as the most effective immunoprevention tools for controlling tuberculosis and, first and foremost, its pulmonary variant (Sierra, 2006). It is evident that their creation requires a deeper insight into the key events at the cell and molecular levels that provide for establishment of immunity (Xing, 2001). In addition, taking into account that one-third of the world population has been already infected with *M. tuberculosis*, we should speak about a “post-infection” vaccine. Such vaccine should meet several requirements, such as induction of a stable specific immunity, minimum side effects, and cost-efficiency suitable for worldwide administration, which is of special importance for the third world countries. A truly effective vaccine should induce a stronger immune response as compared with the immunity induced by natural *M. tuberculosis* infection. In addition, the new vaccine should be able to protect the population cohorts of both previously immunized with BCG and those infected with *M. tuberculosis* and/or HIV and prevent development of pulmonary tuberculosis forms (Martin, 2005).

It is likely that a new vaccine capable of boosting the protective effect of previous BCG vaccination will soon appear. In a more long-term perspective, the vaccine will be designed to replace BCG vaccine. A safe live vaccine involving new *M. tuberculosis* strains will be most likely one of the candidate vaccines (Martin, 2005).

By the mid-1990s, three main directions had established in the development of anti-tuberculosis vaccine, namely, (1) generation of *M. tuberculosis* auxotrophic mutants; (2) BCG improvement via constructing its recombinant analogs secreting cytokines or containing genes of *M. tuberculosis* virulent strains (Nor and Musa,

2004); and (3) design of DNA vaccines (Mollenkopf, et al., 2001) or subunit vaccines using *M. tuberculosis* major proteins (Orme, 1995, 1999; Smith, 2003) or use of recombinant BCG strains for delivery of heterologous antigens (Hanson et al., 1995).

Research in these directions supplemented with use of mycobacterial lipids as a vaccine or adjuvants were continued in the 2000s (Agger and Andersen, 2002; Young and Stewart, 2002; Kumar, et al., 2003; Shi, et al., 2010a, b). Adjuvants are the substances able to increase the specific immune response in vaccination; therefore, adjuvants are selected taking into account a particular type of the induced immune response (humoral, cell-mediated, or mucosal) and the method used for vaccine administration (Vogel, 2000). In particular, cytokines (Xing, 2001), including γ -interferon or its analogs (Azaev, et al., 2004, 2007), can be also used as adjuvants.

An important direction in increasing the efficiency of vaccine is optimization of its form aiming to predominantly induce the cell-mediated immune response (Young, 2000). The intracellular parasites *M. tuberculosis*, *Listeria monocytogenes*, and *Salmonella* (Mollenkopf, et al., 2001; Hall, et al., 2009; Wang, et al., 2009) and several viruses (Xing and Lichty, 2006; Perera, et al., 2009; Zhou, et al., 2010) have been considered as delivery vectors. Use of various nucleoprotein complexes also gives some prospects (Azaev, et al., 2007).

Improvement of the methods for selecting adjuvants and delivery systems gives optimism for the prospects of designing subunit vaccines against tuberculosis that would stimulate the T-cell response (Reed, et al., 2003; Christensen, et al., 2009; Kamath, et al., 2009; Henriksen-Lacey, et al., 2010, 2011).

By the beginning of 2000, there were already several candidate vaccines displaying positive results in preclinical trials in mouse and guinea pig models using aerosol administration (Orme, et al., 2001). Three of these vaccines starting from 2003 are undergoing clinical trials (Orme, 2001, 2005; Reed, et al., 2003). By 2005, various research teams had developed over 200 new candidate tuberculosis vaccines (Haile and Kallenius, 2005); clinical trials have been commenced for some of these candidates. The best results have been obtained with the vaccines involving *M. bovis* recombinant BCG strains, live attenuated *M. tuberculosis* strains, DNA vaccines, and subunit vaccines with new adjuvants (Brennan, et al., 2004). In 2005, preparation to clinical trials of candidate vaccines started in the United States (Rowland, et al., 2005) and the TB Vaccine Cluster project, funded by the European Union Fifth Framework Programme, was launched in Europe. Participants of the European cluster have developed at least 24 candidate vaccines that have demonstrated their efficiency in various animal models (Williams, et al., 2005). The immunological assessment of the vaccines is performed based on the WHO recommendations (Hanekom, et al., 2008).

ANTIGENS PROMISING FOR VACCINE DESIGN

By the beginning of 2000, several variants of subunit vaccines had been obtained; however, their protective effect in trials was in several cases unpredictable and considerably varied. To overcome this disadvantage, it was proposed to design and use the constructs providing for co-expression of recombinant antigens and cytokines or to use the formulations containing both recombinant antigens and cytokines (Sharma and Khuller, 2001; Xing, 2001).

Secreted proteins are the most important group among the *M. tuberculosis* antigens with protective activity (Mustafa, et al., 2002).

Secreted Proteins of M. tuberculosis

Annotation of the genome of *M. tuberculosis* strain H37Rv has demonstrated that it contains 3995 open reading frames (ORFs); however, functional activity has been predicted for only 52% of these ORFs (Camus, et al., 2002). All the detected genes got ordinal numbers preceded by the abbreviation Rv.

Analysis of the *M. tuberculosis* proteome using two-dimensional electrophoresis in combination with MALDI-MS fingerprinting (Jungblut, et al., 1999) has detected at least 1800 cell proteins and 800 secreted proteins.

Presumably, it is reasonable first of all to search the secretory proteins for the antigens promising in designing new vaccines (Skjot, et al., 2000). However, identification of such potential antigens is not a simple problem, since, on the one hand, the precise data on the complete set of the proteins secreted by *M. tuberculosis* in the patient's body is still unavailable and, on the other hand, it is known that their qualitative and quantitative compositions can considerably change depending on the organism response during infection and disease course (Laal and Skeiky, 2005).

The genes coding for proteins that accumulate in the culture liquid during *M. tuberculosis* growth are identified by immunochemical screening of *M. tuberculosis* genomic expression libraries using high titer rabbit antisera obtained in response to immunizing animals with culture filtrate proteins (CFPs). For this purpose, various fractions of *M. tuberculosis* secreted proteins are incubated with blood mononuclear cells of the BCG vaccinees, naive persons, or tuberculosis cases (Surekha, et al., 2005).

The experiment on assessing the effect of ten fractions of *M. tuberculosis* secreted proteins (14–90 kDa) on the lymphocyte proliferation index and synthesis of γ -interferon and interleukin-2 by the peripheral blood mononuclear cells of child donors belonging to the listed groups has demonstrated that the secreted proteins of 30–34 kDa fraction are the most promising for vaccine design since they induce the most pronounced immune response (Surekha, et al., 2005).

ESAT-6 Family

The ESAT-6 family contains low-molecular-weight early proteins encoded by over 20 genes (Brosch et al., 2007). They include not only *ESAT-6* itself (synonyms: *esxA*, *Rv3875*, *MT3989*, and *MTV027.10*), but also the proteins coded for by ORFs *Rv0287*, *Rv0288* (*TB10.4*), *Rv2346c*, *Rv2347c*, *Rv3619c*, *Rv3620c*, *Rv3890c* (*Mb3919c*), *Rv3905c* (*Mb3935c*), and *CFP-10* (*Rv3874*) (Smith, 2003; Brosch et al., 2007), differing in their nucleotide sequences between *M. bovis* and *M. tuberculosis*.

The proteins of this family are highly immune and specific (Skjot et al., 2000). The highest immunogenicity is displayed by *TB10.4* > *CFP-10* ≈ *ESAT-6*, as demonstrated by the level of γ -interferon synthesis by mononuclear cells of peripheral donor blood in response to the contact with the corresponding antigens. Two members of this family—*ESAT-6* and *CFP-10*—are encoded by the *esx-lhp* operon, located in RD1 region of *M. tuberculosis* genome, and are immunodominant T-cell antigens (Okkels, et al., 2003). *TB10.4*, the product of gene *Rv0288*, was first isolated from a low-molecular-weight fraction of *M. tuberculosis* culture filtrate at an early cultivation stage (Skjot, et al., 2000).

The functions of these proteins are yet unknown, but it has been experimentally demonstrated that they are recognized by Th1 cells and can be used in a subunit vaccine (Mustafa and Shaban, 2006). Presumably, the protein *Rv0287*, able to form complex with *TB10.4*, can be also useful for this purpose.

Serine Protease Family

Two MTB32 antigens, A and B, displaying 66% homology, have been detected among the *M. tuberculosis* secreted proteins and identified (Skeiky, et al., 1999). The molecular weight of each protein without a signal peptide is about 32 kDa. Both belong to the serine protease family. The gene *mtb32a* is present in the *M. tuberculosis* genome as a single copy. The corresponding protein is detectable among the secreted proteins of both virulent (H37Rv, Erdman, and clinical isolate CSU93) and nonvirulent (H37Ra) *M. tuberculosis* strains. A homolog of MTB32 was also detected in *M. bovis* BCG. However, MTB32A rather than MTB32B is considered as a candidate for subunit vaccine, since the peripheral blood mononuclear cells of the infected persons do not respond to it. This protein can be also promising for designing diagnostic kits for tuberculosis.

Other Families

CFP32 (*Rv0577*) is a secreted protein, presumably glyoxylase, with a molecular weight of 32 kDa, which is present only in members of the *Mycobacterium tuberculosis* complex (MTC). The CFP32 primary structure is similar in all the MTC members. No polymorphism has been detected by sequencing PCR frag-

ments of the corresponding gene. During cultivation, this protein is detectable by immunoblotting in culture liquid and cell cytosol compartments. Enzyme immunoassay (EIA) has detected the antibodies to CFP32 in the serum of 32% of the pulmonary tuberculosis patients, and the protein itself is detected in the serum of 56% cases. The CFP32 level in serum correlates with the concentration of interleukin-10 (an immunosuppressive cytokine assumed to play an important role in the disease progress) in the serum and displays no correlation with the level of γ -interferon, a key factor in establishment of the protective immunity (Huard et al., 2003). This protein is promising both as a target of anti-tuberculosis drugs and for tuberculosis diagnosing.

Antigen 85 Complex

The proteins of three-component antigen 85 complex—Ag85A, Ag85B, and Ag85C—have been discovered among the proteins secreted by *M. tuberculosis* (Belisle, et al., 1997). All these proteins display a mycolyltransferase activity and catalyze biosynthesis of the most widespread cell wall glycolipids (Dover, et al., 2007; Goude and Parish, 2008), including the virulent cord factor (Elamin, et al., 2009). The proteins Ag85 have close molecular weights of 30–32 kDa (Daniel and Janicki, 1978; Sada, et al., 1990; Laal and Skeiky, 2005). Determination of their spatial structure by X-ray structure analysis (Ronning, et al., 2000) opened the possibility of a purposeful synthesis of enzyme activity inhibitors and design of new anti-tuberculosis preparations. Depending on the cultivation conditions, the proteins of this complex account for 14 to 60% of the secreted proteins (Wiker and Harboe, 1992). It has been shown that recombinant 85A antigen induces the protective immunity, and several subunit vaccines involving this antigen are currently undergoing clinical trials in Africa (McShane, et al., 2005). Of great interest is also antigen 85B, which in combination with other *M. tuberculosis* antigens, in particular, ESAT6, is regarded as a serious candidate for subunit vaccine (Olsen, et al., 2004; Langermans, et al., 2005; Xu, et al., 2009, 2010; Christensen, et al., 2010; Clark, et al., 2010; Shen, et al., 2010; van Dissel, et al., 2010, 2011).

Multi-epitope Proteins

Presumably, it is impossible to construct a subunit tuberculosis vaccine involving only one recombinant antigen. It is more promising to use for this purpose the hybrid proteins containing several antigens or several epitopes of different antigens (Andersen and Doherty, 2005; Luo, et al., 2009; Davila, et al., 2010; Aagaard, et al., 2011). Below are some examples of such artificial proteins.

Hybrid protein ESAT-6–Ag85. The protein obtained by fusing the epitopes of antigens 85A and

ESAT-6 displayed a very good induction of the protective immunity in the guinea pig tuberculosis model (Olsen, et al., 2004). The fused protein Ag85A–ESAT-6 displayed significantly more pronounced immunogenic effect as compared with its components. Being administered in an adjuvant to guinea pigs, this fused protein protected the animals against *M. tuberculosis* infection to the same degree as BCG. Immunization of animals considerably slowed the disease progress after infection with *M. tuberculosis* and extended the lifespan; however, the problem of choosing the most efficient delivery method for the protein still remains (Williams, et al., 2005).

Mtb72f is likely to be among the most promising antigens for designing a subunit vaccine (Mitsuyama, et al., 2003). It has been constructed via a tandem fusion of three epitopes, Mtb32C–Mtb39–Mtb32N (Skeiky, et al., 2004). The corresponding fused gene codes for the polypeptide with a molecular weight of 72 kDa. Immunization of C57BL/6 mice with a “naked” DNA of this gene induces a strong γ -interferon response to the incubation of monocytes with the first two components of this polypeptide (Mtb32C and Mtb39) as well as a strong CD8 response of T cells directed exclusively against Mtb32C. If mice are immunized with the polypeptide Mtb72f, the strength and direction of the immune response depend on the used adjuvant. With the adjuvant AS02A, a moderate γ -interferon response and a weak CD8 T-cell response are induced. In the case of adjuvant AS01B, a powerful immune γ -interferon response and the humoral response to all three components of the polypeptide are induced; in addition, the CD8 T-cell response equivalent to that caused by Mtb72f DNA and directed exclusively against Mtb32C epitope is observed. All the three immunization methods (DNA immunization and immunizations in adjuvants AS01 and AS02) induced in C57BL/6 mice the protective immunity against aerosol infection with *M. tuberculosis* virulent strains. Moreover, which is especially important, the protective immunity was established in guinea pigs (survival over 1 year after aerosol infection), which was comparable in its parameters to that caused by BCG vaccination. In 2004, the subunit AS02-adjuvanted vaccine Mtb72f was among the first candidate vaccines subject to phase I clinical trials in volunteers. A recombinant BCG (72f BCG) expressing this antigen was also constructed (Okada and Shirakawa, 2005).

The most interesting among the *M. tuberculosis* secreted proteins for vaccine design are those that can induce a strong T-cell response and secretion of γ -interferon, which are the integral components of protection against tuberculosis (Ben Amor, et al., 2005; McMurry, et al., 2005; Gao, et al., 2009). It is evident that such proteins should contain T-cell epitopes or binding motifs for MHC class II proteins (proteins of class II major histocompatibility complex).

The advent of computational methods for analysis of nucleotide and protein sequences gave additional possibilities in search for candidate components of multi-epitope vaccines.

The internet-accessible SignalP and Prosite programs allowed the reading frames for putative secreted proteins to be determined in the genome-wide sequences of *M. tuberculosis* strains H37Rv and CDC 1551 (McMurry, et al., 2005). Then the authors used their own patented software to analyze the corresponding proteins for the presence of MHC II binding motifs and found 65000 such sequences, accounting for 5% of the screened 1.3 million primary structures. The 17 sequences with the highest rank were synthesized, and their ability to stimulate the synthesis of γ -interferon and in vitro proliferation of T lymphocytes was studied. For this purpose, the peripheral blood was taken from the healthy donors that yet had a negative tuberculin skin test. In the in vitro experiments, eight polypeptides stimulated lymphocyte proliferation and 15 polypeptides stimulated synthesis of γ -interferon (IFN- γ ELISpot); moreover, one of the latter, MT2281-26-J (WRRRLPLSSALLSFGLLGLPL), induced the synthesis of γ -interferon in specimens of 11 donors of the total 25 (44%). In general, the 15 detected epitopes, especially, MT2281-26-J, are good candidate epitopes for a multi-epitope anti-tuberculosis vaccine (McMurry, et al., 2005).

Another internet-accessible program, ProPred (Mustafa and Shaban, 2006), is also useful in searching for T epitopes. It has found the motifs in ESAT-6, CFP10, and MPT70 that bind over 50% of the panel of class II MHC antigen HLA-DR, comprising 51 polypeptides. Among these motifs are the region of residues 69–77 in ESAT-6, two regions in CFP10 (residues 55–66 and 76–84), and four regions in MPT70 (residues 1–11, 81–95, 124–140, and 192–191). To test these regions for antigenicity, the overlapping peptides were synthesized and their presentation to T cells was studied; thus, it was confirmed that the motifs of residues 69–77 (ESAT-6), 76–84 (CFP10), and 182–191 (MPT70) were recognized by Th1 cells and can be regarded as T epitopes.

An additional source of promising antigens appeared after comparative analysis of the *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG, which detected the ORF-containing regions present only in the *M. tuberculosis* genome (Mustafa, et al., 2002). These regions are referred to as regions of difference (RD). Along with the already identified ESAT-6 and CFP10, the proteins encoded in RD can also contain new proteins with a high immunological reactivity promising as components of subunit vaccines or reagents for tuberculosis diagnosing. Totally, up to 100 proteins are encoded in all regions of difference (Mustafa, 2005). The most immunologically reactive of these proteins could be good candidate vaccines as well as promising components for specific test kits for diagnosing tuberculosis.

One of the directions in this field is production of protein recombinant analogs or overlapping synthetic peptides and assessment of their serological and Th1-cell reactivities. Thus, several antigens encoded in RD1 region and promising for specific diagnostic kits intended for low endemic regions and candidate vaccines have been detected (Mustafa, 2005). Among these antigens are ORF14 and ESAT6, which are present in all the virulent *M. tuberculosis* strains and determine the degree of virulence (Okkels, et al., 2003), as well as CFP10, PE, and PPE.

Specialized software aimed to detect antigenic motifs able to bind several HLA proteins are widely used in designing vaccines. Such motifs and the antigens where they are contained, recognized by T cells, can be promising for construction of subunit vaccines for prevention of tuberculosis and test kits for its diagnosis (Mustafa, 2005).

ADMINISTRATION ROUTES OF ANTI-TUBERCULOSIS VACCINES

There are two main routes used for administering the live BCG vaccine, namely, oral and parenteral (intracutaneous or subcutaneous). Initially, BCG was intended for exclusively oral administration. The *M. bovis* BCG strain Moreau Rio de Janeiro for oral vaccination of children and adults is still commercially available in Brazil (Badell, et al., 2009).

The post-vaccination complications depend on the BCG administration route. Cervical lymphadenitis and granulation otitis may develop in the case of oral immunization. The cause of complications in vaccinated infants is penetration of the liquid vaccine from the nasopharynx via the eustachian tube into the middle ear, especially in possetting. Use of intracutaneous administration caused new complications as local inflammatory reaction on the skin at the site of vaccine injection or in the regional lymph nodes.

There were two reasons for rejecting the practice of oral BCG administration: first, because of a drastic (10- to 100-fold) decrease in the BCG viability caused by a low pH of the gastric juice and action of digestive enzymes, which required increased vaccine doses, and, second, due to a high rate of cervical lymphadenitis (Bloom and Fine, 2002).

Currently, a subcutaneous BCG injection is widely used (Badell, et al., 2009). New candidate tuberculosis vaccines are also mainly intended for parenteral administration (other than through the digestive tract).

However, *M. tuberculosis* enters the human body mainly through the airway penetrating via the mucosa or lung mucous membrane. The vaccines intended for inducing the immune response at the level of mucosae and the administration via airways are undoubtedly promising for vaccination against tuberculosis, since they stimulate the immune response directly at the introduction of the active substance (Hall, et al., 2010).

In particular, it has been demonstrated that only 10 BCG bacteria administered to guinea pigs as an aerosol provide the same or even stronger immunity as compared with considerably larger vaccine doses injected intracutaneously or subcutaneously. The experiment with aerosol rhesus macaque model and BCG or its cell wall material demonstrated a sufficient protection level and was not accompanied by conversion of tubercular test, i.e., any transition of previously negative test into positive was undetectable, which demonstrated the preservation of immunization status. In addition, it was found that an aerosol administration of BCG vaccine to healthy volunteers caused no side reactions (Bloom and Fine, 2002).

An important argument in favor of mucosal vaccines is the absence of traumatization of vaccinees, inevitable in the case of injection administration.

Taking into account that both the local and systemic immune responses are tightly connected and that the mucosal vaccination minimizes the risks associated with skin damage during vaccination, it is most likely that mucosal vaccine will gradually replace the injection variants (Reljic, et al., 2006; Xing and Lichty, 2006).

Biotechnological methods have brought about new possibilities for administration of subunit tuberculosis vaccines, in particular, the possibility of constructing transgenic plants and their use as a delivery tool for vaccines (Salyaev, et al., 2010).

TRANSGENIC PLANTS AS PRODUCERS OF "EDIBLE" VACCINES

When designing new generation vaccines involving recombinant antigens, the search for highly efficient and cost-effective expression systems for production of the corresponding protein antigens remains topical. Currently, *E. coli*, *S. cerevisiae*, as well as human and insect cell cultures are used for this purpose (Sourrouille, et al., 2009). However, these systems are not free from disadvantages connected with posttranslational modifications of recombinant proteins, the time required for synthesizing the target product, limited proliferative potential, high price of the culture media, etc.

Genetically modified plants open new prospects for producing recombinant pharmaceutical proteins (Ma, et al., 2005; Hefferon, 2010), since such plants can serve as less expensive and safer source of recombinant proteins as compared with the traditional expression systems involving bacteria, yeasts, as well as insect and mammalian cell cultures (Menkhaus, et al., 2004; Boehm, 2007; Frutos, et al., 2008; Sourrouille, et al., 2009).

Plants used as expression systems for accumulation of recombinant proteins of pharmaceutical importance are most attractive due to many circumstances. First and foremost, plant tissues exclude any possibility of contamination with the agents pathogenic for mammals, such as viruses and prions. Plant cells pro-

vide for a correct posttranslational modification of recombinant protein characteristic of eukaryotic cells as well as its assembly and folding (Ma, et al., 2005).

The recombinant proteins expressed in plant tissues can be directed to various compartments of the plant cell (vacuoles or lumen of the endoplasmic reticulum) as well as into the apoplast and various plant organs (seeds, tubers, fruits, etc.). Correspondingly, the recombinant proteins in plant tissues can be preserved for a long time (for months and years) without any changes and decrease in biological activity (Sourrouille, et al., 2009).

Of great importance is also the fact that the agrobiological methods for cultivation of agriculturally important plant species developed so far as well as the seed production systems for particular cultures make plants attractive as biological factories for producing proteins of medical importance.

Note that the plants that can be eaten without heat processing are a ready product for disease prevention and treatment. Such plants where recombinant bacterial antigens are synthesized and accumulated in certain tissues are attractive as vaccines and referred to as "edible" vaccines (Mason, et al., 2002; Rybiski, 2008; Tecson, et al., 2008; Yusibov and Rabindran, 2008). Moreover, transgenic plants are convenient models for designing new alternative routes (oral and intranasal) for delivery of recombinant proteins into the body of warm-blooded beings.

Induction of Immune Response Using "Edible" Vaccines

The mechanism of immunization with edible vaccines is based on the antigen presenting ability of peritoneal macrophages in the small intestine of mammals. In the intestine, a foreign protein displaying antigenic properties is recognized by specialized M cells, which are abundant in the mucosal epithelium. M cells transport the captured antigen to the peritoneal macrophages and B lymphocytes located in the lymphoid structures of small intestine (Peyer's patches). Presentation of an antigen on the surface of antigen presenting cells activates T helper lymphocytes, which in combination with the antigen activate B lymphocytes. Differentiated B cells leave the mucosal lymphoid follicles to reach mesenteric lymph nodes via the general circulation; there they mature and transform into plasmatic cells, synthesizing the major immunoglobulin of the mucosal secrete, immunoglobulin A (sIgA), the so-called marker of "local immunity".

The plasma cells are able to migrate again to respiratory, gastrointestinal, and urogenital mucosae. The secretory immunoglobulin blocks the adhesion of pathogens to the mucosal surfaces, preventing their penetration into the subjacent tissues and thereby providing the barrier function of mucosae as the first line of immunological protection on the way of pathogens.

Microbial antigens are among the active stimuli for the sIgA synthesis in intestine.

Taking into account that mucosal vaccination stimulates both the local immune response at the level of mucosae and the general immune response of the body, edible vaccines is among the promising directions in obtaining cost-effective vaccines (Shchelkunova and Shchelkunov, 2008). So far, the transgenic plants of tobacco, tomato, lettuce, turnip rape, arabis, and turnip carrying the genes that control synthesis of various antigens and antibodies have been constructed.

Note that the genetically modified plants with nuclear transformation i.e., the plants where a foreign gene is inserted into the nuclear genome, are mainly used for production of recombinant proteins and designing of edible vaccines. In addition, the plastomic plants with the foreign gene delivered to the chloroplast genome are used as well as the agroinfiltration method, based on a transient expression of foreign genes in plant cells. More detailed information about the state of activities on designating edible vaccines using genetically modified plants is available in the reviews of the *Expert Review of Vaccines* special issue (vol. 9, no. 8, 2010) and in the cited papers (Rukavtsova, et al., 2006; Sourrouille, et al., 2009; Hefferon, 2010).

Edible Vaccines against Tuberculosis

As has been demonstrated, the delivery of *M. tuberculosis* antigens through mucosa (oral or intranasal) can induce the protective immune response. This fact gave new birth to oral immunization against tuberculosis with the help of transgenic plants. Accumulation of antigens in the plant tissues eaten without any preliminary cooking is of great interest for designing new generation vaccines, since the cellulose envelope of plant cell can act as a microcapsule and protect the contents from a damaging impact of the gastrointestinal pH and enzymes. Moreover, to prevent immunological tolerance in the intestinal mucosae, it is possible to produce the transgenic plants that would contain not only the genes encoding the envelope proteins of a pathogen, but also the genes of the proteins acting as adjuvants. In particular, a fused variant of the *M. tuberculosis* gene *esat6* and the gene coding for heat-labile enterotoxin B subunit (LTB) of *E. coli* enterotoxigenic strain were introduced into arabidopsis plants (Rigano, et al., 2004).

Currently, a wide range of antigens acting as targets of the immune responses to *M. tuberculosis* are obtained in various laboratories worldwide. Among such proteins are the *M. tuberculosis* secreted protein family Ag85, the members of which, Ag85A and Ag85B, can be used in the new generation vaccines. Another promising target is the protein ESAT6. The possibility of constructing an edible vaccine simultaneously containing two antigens, for example, ESAT6

and Ag85B, accumulated in transgenic lettuce plants (Matveeva, et al., 2009; Floss, et al., 2010) also attracts attention of researchers. Plants can be efficiently used for biosynthesis of *M. tuberculosis* antigens using a transient production of target proteins with the help of plant viruses as vectors (Zelada, et al., 2006; Dorokhov, et al., 2007). A technology for constructing the transgenic plants producing the *M. tuberculosis* antigens ESAT6 and CFP10 as well as human deltaferon as an immunomodulatory mediator is now developed; in this method, the corresponding genes are fused in one reading frame and transferred by agrobacterial transformation into the genome of carrot plants (Tat'kov, Deineko, et al., unpublished data).

Rigano, et al. (2006) demonstrated successful vaccination with the transgenic plants accumulating *M. tuberculosis* antigens in their tissues. The authors have shown a stimulation of γ -interferon synthesis by mouse CD4⁺ cells, which suggests the induction of a Th1 type of the immune response after eating transgenic plants.

CONCLUSIONS

Abundance of multiple and extended drug resistant *M. tuberculosis* strains makes ever more topical the problem of improvement of the anti-tuberculosis vaccine. According to the literature data, one of the promising directions is construction of artificial multi-epitope antigens and selection of the optimal delivery route into the human body. Transgenic plants biosynthesizing the corresponding antigens provide most promising prospects for revival of oral vaccination against tuberculosis. Such plants not only allow the necessary antigens to be produced with minimum expenditures, but also provide an optimal solution for their delivery to the sites of immune response formation, since the plant cells can act as a natural "nanocapsule" for the antigen to pass through the gastrointestinal tract and induce the protective immune response.

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