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Defining B Cell Epitopes of Ovalbumin for the C57BL/6 Mice Immunized with Recombinant *Mycobacterium smegmatis*

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Recombinant *Mycobacterium smegmatis* expressing ovalbumin was used to immunize C57BL/6(H-2^b) mice, and the humoral immunity against recombinant ovalbumin was analyzed. Antibodies were purified by denatured ovalbumin-conjugated affinity chromatography. The epitopes of the antibodies were screened with a random peptide library displayed on the tip of fUSE5 filamentous phage pIII minor coat proteins. Two peptides, IRLADR and SPGAEV, were selected predominantly by the recognition of purified antibodies using biopanning methods.

The composition of the peptide sequence with the primary structure of OVA revealed that the peptide sequence analogizes to INEAGR, part of the ^{323}ISQAVHAAHAEINEAGR^{339} sequence previously reported as the antigenic determinant for murine B and also Th cell epitopes (I-A^d binding). Also, the structures of these mimotopes obtained from restrained molecular dynamic computations resulted in the formation of a β-turn proven to be a secondary structure of the parent peptide within the ovalbumin molecule, enabling us to confirm the structural similarity.

This study demonstrates that immunization with recombinant *M. smegmatis* can generate neutralizing antibodies identical with those induced by the administration of natural antigenic proteins and supports the potential use of mycobacteria as vaccine delivery vehicles.

Keywords: Epitope, Mimotope, Ovalbumin, Recombinant *Mycobacterium smegmatis*, Recombinant vaccine.

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Introduction

Recombinant bacterial vectors have recently been utilized as agents for inducing both humoral and cellular immune responses with a view towards vaccine development. These recombinant microbes include live, avirulent bacteria such as attenuated *Salmonella* (Soo *et al.*, 1998), viruses such as adenovirus and poliovirus (Jung and Bae, 1998), and intracellular eneric pathogens, Gram-negative *Shigella flexneri* (Phalipon and Sansonetti, 1995) and *Yersinia enterocolitica* (O'Gaora *et al.*, 1990), and Gram-positive *Listeria monocytogenes* (Shen *et al.*, 1995). Also non-pathogenic bacteria including commensal organisms, strains of *Lactobacillus* spp. (Gerritse *et al.*, 1990; Norton *et al.*, 1994), *Bifidobacterium* spp. (Rossi *et al.*, 1998) and *Streptococcus* have been constructed to express heterologous vaccine antigens (Oggioni *et al.*, 1995). *Mycobacterium bovis* strain *Camette-Guerin* (BCG) and *Mycobacterium smegmatis* (MS) (Aldovini and Young, 1991; Stover *et al.*, 1991; Hetzel *et al.*, 1998) are also attractive as live recombinant vaccine vehicles. However, they are poorly characterized at the molecular level regarding the mutations responsible for attenuation, require the administration of multiple doses to induce optimal immunity, replicate poorly *in vivo*, and induce only modest cell-mediated immune responses. These difficulties needing to be addressed have led to the search for more effective live attenuated carriers (Cho *et al.*, 1998).

The purpose of this work was to evaluate the efficacy of mycobacterial immunization in the induction of neutralizing antibody (Ab) to a model pathogen, ovalbumin (OVA), expressed. We chose *M. smegmatis* as a host microbe because it grows more rapidly than other tubercle bacilli in culture and has not been established as a pathogen. To confirm the ability to induce a desired humoral immune response by recombinant *M. smegmatis* (rMS) immunization, epitope comparison is a solid

criterion as it requires a complementary paratope for its operational recognition (Van Regenmortel, 1989). Moreover, as the number of peptide vaccines has grown, the concept of an epitope has gained favor. The delineation of epitopes can be achieved by antigenic cross-reactivity studies or by X-ray crystallography. Both approaches require specific criteria for deciding which residues of the antigen are in contact with the paratope and are functionally part of the epitope. Recent alternative techniques for epitope identification have used large libraries of random peptides displayed on the surface of filamentous bacteriophage (Scott and Smith, 1990; Carcamo *et al.*, 1998) or are chemically synthesized (Stevens *et al.*, 1998). Currently, this technology has become very popular (Dybwad *et al.*, 1995; D'Mellow *et al.*, 1997; Murray *et al.*, 1997).

Ovalbumin (OVA) is biochemically well characterized (Milstine, 1968; Nisbet *et al.*, 1981; Stein *et al.*, 1991; Jeoung and Yu, 1999) and has universally been used in immunological studies as a model antigen (Bazin and Bernadette, 1976; Fremont *et al.*, 1995; Bennett *et al.*, 1998). Previous works have also defined the peptide epitopes for anti-OVA Ab from mouse or rabbit (Johnsen and Elsayed, 1990), helper T-cells (Buus *et al.*, 1987), and cytotoxic T-lymphocytes (Rotzschke *et al.*, 1991). A parallel conclusion of the antigenic determinant for the IgE isotype in human patients allergic to egg was assigned as well (Johnsen and Elsayed, 1990). In this study, we identified several mimotopes of OVA with the phage-displayed peptide library, the majority of which possess a central motif present within the peptide reported previously as the epitope for human serum, rabbit, and mouse anti-OVA Ig isotypes. The results indicate that it is possible to induce anamnestic responses and protective immunity by vaccination with recombinant mycobacteria expressing target antigen.

Materials and Methods

Recombinant *Mycobacteria* culture *M. smegmatis* strain *mc²155* was transfected with plasmid pEYO by an electroporation method and then grown at 37°C with vigorous shaking in tryptic Soy broth (Difco, Detroit, USA) supplemented with 1% glucose, 0.05% Tween 20, and 20 μg/ml kanamycin sulfate. The plasmid pEYO was constructed by subcloning the cDNA of the chicken ovalbumin gene from the plasmid pOV_{230} (McReynolds *et al.*, 1978) into the *Escherichia coli*-mycobacterial shuttle vector pYUB12 (Snapper *et al.*, 1988). The resulted plasmid pEYO had the kanamycin-resistance gene, origin of mycobacterial replication, as well as the origin of *E. coli* replication.

Immunoblotting of recombinant *Mycobacteria* The recombinant OVA in rMS cells were detected by Western blot analysis on bacterial cultures. Whole cell heat-denatured extracts were subjected to 12% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and probed with rabbit anti-OVA Ab (Promega, Madison, USA).

Immunization of mice C57BL/6 mice (5–8 wk, female) were immunized with rMS by intraperitoneal inoculation of $1–10 \times 10^7$ bacilli in 0.2 ml phosphate-buffered saline (PBS) and boosted twice at 3-week intervals under the same condition.

Serum antibody purification Mice were bled from the tail or eye and sera were separated and pooled. Serum (1.5 ml) was diluted in PBS (total vol. 3 ml) and passed through an OVA-conjugated affigel-15 affinity column activated with succinylamide (Bio-Rad, Madison, USA). After conjugation, ligand OVA was denatured with 6 M urea, and washed thoroughly with PBS containing 0.2% Tween 20. Non-specifically bound serum proteins were flushed out with 0.3 M NaCl-10 mM Tris·HCl (pH 7.2) buffer, and then anti-OVA Ab was eluted with 0.1 M glycine-HCl buffer (pH 2.2). The glycine-buffered fractions containing anti-rOVA antibodies (1 ml each) were neutralized to pH 7 by the addition of 200 μl of 1 N NaOH, and dialyzed against PBS containing 0.02% NaN_3. Finally, the Ab solution was concentrated with Centricon-10 (Amicon, Beverly, USA), and stored at 4°C. Purified anti-OVA Ab was biotinylated with NHS-LS-biotin (Pierce, Rockford, USA) for biopanning of phage.

Mimotope screening The phage-display peptide library was amplified from a primary library consisting of approximately 6.4×10^8 independent transformants (Scott and Smith, 1990), and was generously given to us by Prof. G. P. Smith of the University of Missouri, USA. Screening phage-displayed random peptide libraries to identify peptide mimotopes for anti-OVA Ab was conducted as previously described (Sparks *et al.*, 1996). In brief, petri dishes (Φ35 mm) were coated with 1.5 ml of 0.1 mg/ml streptavidin overnight and then emptied and blocked for 2 h with 5 mg/ml dialyzed BSA (Sigma, St. Louis, USA). During the first round of biopanning, 1 μg of biotinylated Ab was loaded on the streptavidin-coated petri dish for 2 h, rinsed three times with TBS-Tween (10 mM Tris·HCl, 150 mM NaCl, 0.5% Tween 20, pH 7.4), and then incubated in a 0.4 ml volume containing $\sim 10^{10}$ phage for 4 h at 4°C with gentle agitation. Ab-bound phage were eluted from the dish by gentle shaking for 10 min in 0.5 ml of 0.1 M glycine-HCl buffer (pH 2.2) containing 1 mg/ml BSA followed by neutralization with 2 M Tris. From the 2nd to 7th rounds of biopanning, biotinylated Ab (0.8 to 0.1 μg) was incubated in a 100 μl volume of phage solution overnight at 4°C, and then the Ab-bound phage were captured by streptavidin on the petri dish.

DNA sequencing Single-stranded phage DNA was prepared from overnight cultures of K91Kan cells infected with individual colonies, and DNA was then sequenced with the dideoxy-mediated chain termination method, using [α-^{35}S]dATP (Amersham, Buckinghamshire, UK), a DNA sequencing kit (USB, Cleveland, USA), and primer (5′-CCCTCATAGTTAGCGTAACG-3′).

Homology comparison Amino acid sequences were analyzed using the sequence analysis software, DNASIS™ for Windows ver. 2.5 (Hitachi Software Engineering Co., San Francisco, USA). Final alignment and editing were performed manually.

Computer simulation Molecular dynamics computations and energy minimization were carried out with XPLOR 3.8 to address the possible explanation of the affinity associated with mimotopes. A recently obtained X-ray crystal structure was used as an initial structure for detailed computations, including the sequence substitution with mimotopes. After pre-equilibration of the structures was made, high temperature molecular dynamics (500 K, 1000 steps), slow cooling from 500 K to 300 K, and conjugated gradient energy minimization were subsequently carried out.

Results

We have been studying OVA as a model antigenic protein and evaluating its immunogenicity when the OVA gene is expressed in Mycobacteria. To this end, cDNA of the chicken OVA gene with a mycobacterial hsp70 promoter at its 5′-end was inserted into a mycobacterial plasmid and transfected into *M. smegmatis*. The expression level of OVA in rMS was about 1% of total bacterial protein, when bacterial lysates were examined by Western blot (Fig. 1). This level was equivalent to 50 ng of rOVA/5 × 10^7 bacilli, measured by ELISA with goat anti-mouse IgG. All the mice that had been immunized three times with rMS

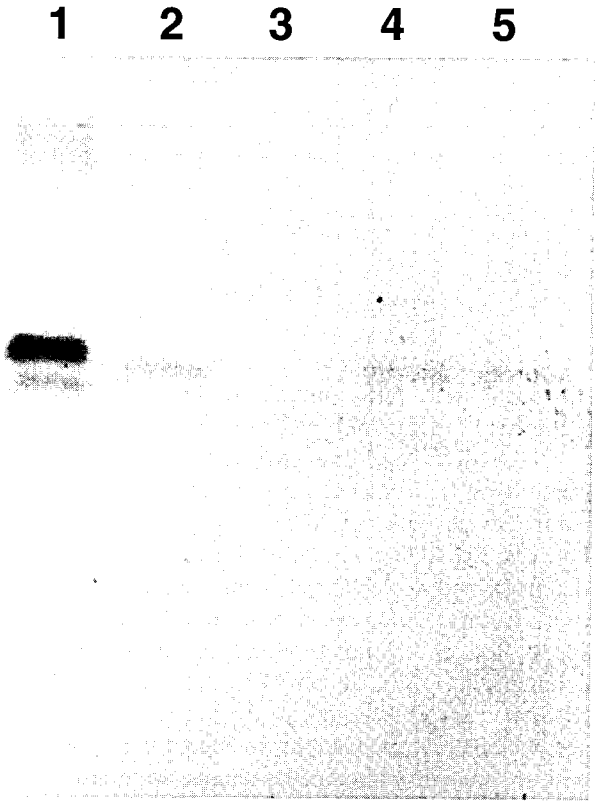

Fig. 1. Western blot of OVA expressed by recombinant mycobacteria. Electrophoresed mycobacterial lysates were blotted and ovalbumin was detected by alkaline-phosphatase conjugated rabbit anti-ovalbumin antibody. Lane 1, chicken ovalbumin (40 ng); lane 3, control MS; and lanes 2, 4, 5, rMS.

intraperitoneally produced anti-OVA Ab successfully. Sera from five immunized mice were pooled and anti-rOVA Ab was then purified. In order to dislodge the antibodies from their conformational epitopes, the OVA ligand was first denatured with 6 M urea, and then all chromotography buffers were supplemented with 0.2% Tween 20. Thus obtained Abs were investigated to define epitopes by the biopanning technique of phage-displayed random peptide libraries. A library of peptides of hexa-amino acid residues fused to the major coat protein pIII of phage was chosen as the epitope source. As biopanning is aimed at identifying ligands with high affinity, the strategy adopted for selecting peptides on phage using anti-rOVA Ab was comprised of multiple rounds of affinity selection with decreasing concentrations of the Ab. We performed seven rounds of biopanning and picked twenty phage clones at random from each of the fourth through seventh rounds; a total of 80 clones of fd-phage DNA were sequenced. The hexa-amino acid sequences of the peptides deduced from the above DNA sequences are shown in Table 1.

Alignment of these hexapeptide sequences with the OVA sequence was performed using the DNASIS homology sequence analysis software. If a minimum of three residues were identified as matching with the OVA sequence, the peptide was admitted to the homologous mimotope group. A total of eighty peptides were sorted into five groups as shown in Table 1. If more than one phage clone had homology to OVA at the same specific region, they were classified into groups 1, 2, and 3 according to the shared OVA motifs, ^{61}KLPGFG^{66}, ^{98}SFNLSG^{103}, and ^{334}INEAGR^{339}, respectively. The clones in group 4 had homology to OVA individually without any comrades. Group 5 clones had no obvious linear homology with the other selected peptides or with OVA. Twenty phage clones obtained from the 4th round displayed distinct mimotope peptides with each other. However, specific mimotopes were reiterated and overlapped from the 5th round of biopanning. The mimotopes HLPAGF (group 1) and MLVSRL (group 4) were matched four amino acids. Nevertheless, these phage clones faded away by repeated biopanning. Eventually, two phage species carrying IRLADR (group 3) and SPGAEV (group 5) were representative at the 7th round of biopanning (Table 2). The hexapeptides carried by 43 fusion phages at the 6th and 7th round of biopanning are shown in Table 2. Among the 43 random positive clones, 14 clones carried the IRLADR sequence that had a significant homology to the ^{334}INEAGR^{339} sequence of OVA. Eleven clones carried a non-homologous SPGAEV sequence. Thirteen less frequently selected positive phages displaying LVYLAR, VSNPDQ, or STSGAY sequences also had linear homology with OVA that belonged to group 4. Other clones not listed in Table 2 were picked up only once at the 6th and 7th rounds of biopanning. Interestingly, the peptide SPGAEV is a reverse-ordered retro-structure of VEAGPS

Table 1. Phage-displayed peptides selected from biopanning with affinity-purified polyclonal anti-rOVA antibodies. Results of biopanning rounds 4 through 7 were classified according to their homologies to OVA. Twenty phage clones were chosen at random from each biopanning round and the amino acid sequences of mimotopes were deduced from their DNA sequences. Residues identified as matching the OVA sequence are highlighted in boldface. The corresponding OVA sequence is given and numbered as in the sequence of the chicken OVA.

	Group 1 ^{61}KLPGFG^{66}	Group 2 ^{98}SFNLSG^{103}	Group 3 ^{334}INEAGR^{339}	Group 4 OVA RELATED	Group 5 UNRELATED	N[a] (ea)
4th	**H**LPA**GF** **HLPGF**W		**IR**LA**DR** **VEAGP**S	LVYLAR MLV**SRL** **STSGAY** VSNPDQ WNTSLL	EAKGPI FRVSAR LGFHRP L?FSAG[b] RFHLPQ SPGAEV SPRVRT WSIALP YTLISY	18
5th	**H**LPA**GF** IWL**GFG**	RAV**LSG**	**IRLADR**	LVYLAR LWNTAS WNTSLL STSGAY	EAKGPI GRKSFL HRKPSD LGFHRP SPGAEV	13
6th	**HLPAGF**	RNV**LSG**	**IRLADR**	LHDKSS LVYLAR VSNPDQ	RQRFIV VLRRCC SPGAEV	9
7th			**IRLADR**	LVYLAR STSGAY VSNPDQ	SPGAEV	5

[a] The number of individuals of different peptide sequences among 20 unintentionally chosen phage clones. Identical peptides harboring phage clones that were sequenced repeatedly were counted as one.

[b] ?, DNA sequence was partially illegible because of multiple bands.

Table 2. Amino acid sequences carried by 43 fusion phages analyzed from biopanning rounds 6 and 7. The best matches found for the peptide motifs with the sequence of OVA and the position of the matching residues are shown. Bold type indicates the matching residues with the motif. Among the 43 clones, 38 clones are in the table and the remaining 5 phage clones, which were found individually at the 6th or 7th biopanning rounds are shown in the footnote.

Amino acid sequences[a]	% Frequency (No_i /No_T)[b]		Peptide in OVA
I R L **A** D **R**	33	(14/43)	^{334}INEAGR^{339}
S **P G A E** V	26	(11/43)	^{334}INEAGR^{339}
L **V Y L A R**	12	(5/43)	^{279}KVYLPR^{284}
V **S N P D Q**	9	(4/43)	^{204}ESKPVQ^{209}
S T S G A Y	9	(4/43)	^{98}SFSLAS^{103}

[a] HLPAGF, RNVLSG, LHDKSS, RQRFIV, and VLRRCC were found only once at the 6th or 7th round of biopanning.

[b] No_i, number of identical clones; No_T, total number of clones (43 ea.) analyzed.

(group 3, Table 1) which was found in the 4th round but disappeared in later rounds. In order to identify the structural relationships between OVA sequence ^{323}INEAGR^{339} and the two IRLADR and SPGAEV mimotopes, molecular dynamics and energy minimization computations with the XPLOR3.8 program were carried out. The IRLADR and SPGAEV structures obtained from the restrained molecular dynamic computations enabled the structural comparison with INEAGR, as shown in Fig. 2. Both of the mimotopes resulted in similar side chain orientations (I, A, R for IRLADR and EAG for the SPGAEV; see Discussion) to the authentic INEAGR. The secondary structural features, including the β-turn together with the overall mimotope structures, were also similar to those of INEAGR as shown in Fig. 2. Such structural similarity might be the basis for the antigenic mimicry for the retro-isomers SPGAEV and VEAGPS. Therefore, the binding behavior of representatives of the two dominant phage species gave a match of three amino acids with the OVA sequence ^{334}INEAGR^{339}.

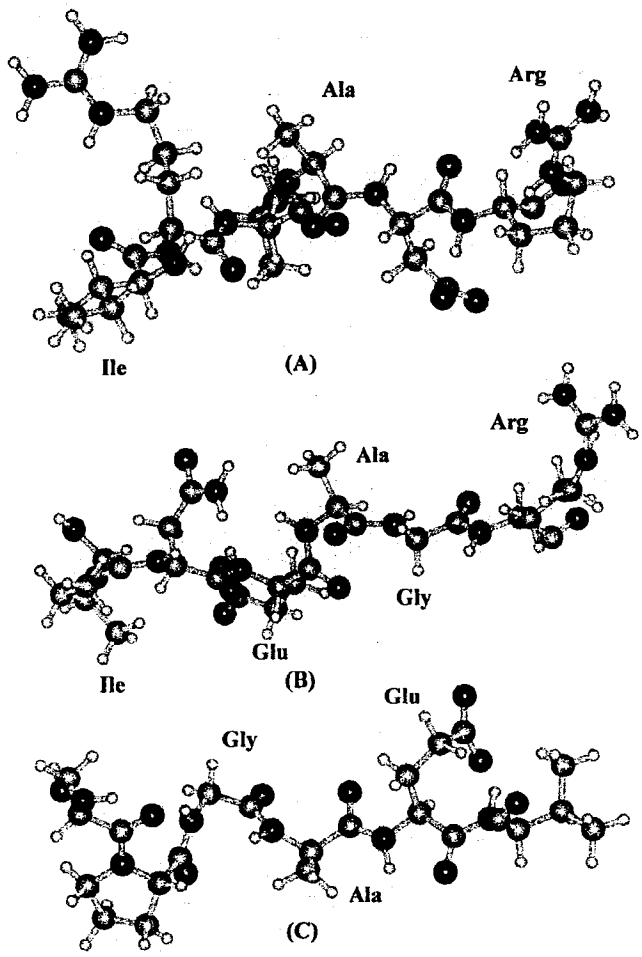

Fig. 2. Ball and stick model drawing of peptide sequences of IRLADR (A), INEAGR (B), and SPGAEV (C) exhibiting the molecular skeleton and orientation of side chains. B and C are the results from the restrained molecular dynamic computations. The side chain orientations of the Ile, Ala, Arg residues are similar in the A and B structures. Mirror image and molecular orientation shows that retro-isomer formation is possible.

Discussion

The humoral response to rMS expressing OVA was induced in H-2b C57BL/6 mice and the affinity-purified anti-rOVA antibodies were investigated to define the epitope by the biopanning technique of the phage-displayed random peptide library. Screening of the phage-displayed random peptide libraries has recently emerged as a powerful technique for probing antigen-Ab interactions. It may be explained by the relative intrinsic affinity of Ab-epitope binding and also their concentrations even though there is no information on the strict correlation between the strength of antigen-antibody interactions.

Of the phages isolated by biopanning against the polyclonal Ab preparation, 25 of the 43 selected clones (Table 2) could be assigned to ^{334}INEAGR^{339} of OVA, a consensus motif, on the basis of sequence similarities. Structures of the IRLADR and SPGAEV sequences obtained from the restrained molecular dynamic computations enabled a structural comparison with ^{334}INEAGR^{339}. The most abundant sequence among the analyzed phage was IRLADR with three matching and three mismatching residues to INEAGR. The matching three residues, Ile, Ala, and Arg, were located on the same topological space as shown by computer aided structural simulation as shown in Fig. 2, (A) and (B). Interestingly, the second most abundant sequence SPGAEV in the reversed order of the VEAGPS sequence was expected to be a better mimotope of INEAGR. The reversed GAE sequence also showed the same topological localization on the basis of structural simulation as shown in Fig. 2, (B) and (C). When a peptide analogue in which the direction of the peptide bond is reversed, by aligning the residues in the reverse order of that in the parent molecule, a retro-structure is obtained. In such a retro-structure, the side chains are oriented in a manner similar to that in the D-enantiomer (Carver *et al.*, 1997). In the case of SPGAEV, however, because of the simplicity of the side groups of glycine (–H atom) and alanine (–CH_3 group), GAE and EAG were not in mirror images even though they shared inherently chiral secondary structure elements. Results in similar side chain orientation (Ile, Ala, Arg) and possible formation of retro-isomer for the reversed amino acid sequences (Gly, Ala, Glu), as shown in Fig. 2, might be representing the high binding affinity of the current experiment.

A 17-amino acids tryptic peptide of chicken OVA, ^{323}ISQAVHAAHAEINEAGR^{339} (OVA323-339), was successful in binding human IgE from egg-allergic patients, rabbit IgG, and mouse IgG, indirectly demonstrating B-cell recognition of these regions in humans, rabbits and mice (Shimonkevitz *et al.*, 1984; Johnsen and Elsayed, 1990). Buus *et al.* (1987) had previously shown that the very peptide was also a Th cell epitope. The data from the N- and C-terminal deletions of OVA323-339 led to the conclusion that the region of the OVA peptide involved in I-A^d interaction was encompassed within residues 327–332 (Buus *et al.*, 1987). Our results strongly suggest that the C-terminal of the OVA 323–339 including ^{334}INEAGR^{339}, is the native B-cell epitope of the natural antigen and this is a rational conclusion together with the N-terminal part as a Th cell epitope.

Mice intraperitoneally immunized with rMS produced anti-OVA Ab whose epitope was exactly at the same antigenic site recognized by the natural antigen-induced Ab. The antigenic determinants or epitopes of a protein correspond to those parts of the molecule that are specifically recognized by the binding sites or paratopes of certain immunoglobulin molecules. Epitopes are thus relational entities that require complementary paratopes for their operational recognition. Understanding the

immunological responses against chicken OVA expressed in mycobacterial cells was a crucial goal of this project aimed at vaccine development.

In conclusion, the use of recombinant mycobacteria-based vaccines may be able to effectively deliver heterologous antigens and induce anamnestic responses.

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