UNIVERSIDAD DE BARCELONA
FACULTAD DE MEDICINA

DESARROLLO DE UNA VACUNA PREVENTIVA CONTRA EL VIH, BASADA EN BCG RECOMBINANTE

TESIS DOCTORAL: ELIAS B. PEZZAT SAID
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IX. MANUSCRITOS EN PREPARACIÓN Y COMUNICACIONES ESCRITAS ACEPTADAS Y PRESENTADAS EN CONGRESOS INTERNACIONALES

CONFERENCIA INTERNACIONAL SOBRE VACUNAS VIH. Laussane, septiembre 2004

Alpha-antigen promoter from *M.tuberculosis* and lysine gene complementation prevent the disruption of heterologous HIV-1 gp120 gene expression by genetic rearrangements in BCG strain lysine auxotrophic. A critical issue for recombinant BCG based HIV-1 vaccine development

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Background: A promising approach for an HIV vaccine is *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) as bacterial live recombinant vaccine vehicle. In this study, we have evaluated the disruption of heterologous HIV-1 gp120 gene expression by genetic rearrangements in BCG host Pasteur strain using a replicative (pMV261) vector. We have also employed the replicative (pJH222) vector in BCG host strain lysine auxotrophic complemented with a plasmid carrying a lysine complementing gene from *M. smegmatis* to compare the HIV-1gp120 gene expression and plasmid stability *in vivo*. **Methods:** The entire DNA coding sequence of HIV-1 gp120 (HXBc2 strain) (from N. Letvin) was amplified by PCR and cloned into different *Escherichia coli- Mycobacteria* shuttle vectors (from B.Bloom and W.Jacobs) under the regulatory control of BCG *hsp60* promoter (pMV261) and *M. tuberculosis* alpha-antigen promoter (pJH222). The coding sequence was fused to *M.tuberculosis* 19k-Da lipoprotein signal sequence (pJH222). BCG strains were transformed by electroporation and transformant BCG screened onto 7H10 medium plates containing kanamycin (25 ug/ml). Lysates of recombinant BCG were separated by 10% SDS-PAGE and analyzed for expression by Western blot analysis using monoclonal antibody directed to influenza hemagglutinin epitope. The mycobacterial plasmid DNA was used as template for PCR analysis and DNA sequencing. **Results:** 12 out of 14 rBCG:HIV-1gp120(261) colonies screened

showed a partial deletion in the DNA coding sequence and 2 did show a total deletion. On the other hand, 10 out of 10 rBCG:HIV-1gp120(222) colonies screened kept the fragment DNA intact (DNA sequencing confirmation). The partial deletion (900 bp) corresponds to gp120 core containing 3 glycosilations sites and V3, V4 and V5 regions of HIV-1 env. All rBCG mutants kept the ORF and one of them was analyzed by Western blot detecting a band of 25-KDa (truncated protein) and the expected 67-KDa of the rBCG:HIV-1gp120(222) recombinant protein. Strikingly, the DNA sequence coding for HIV-1 envelope immunodominant CTL epitope (P18 peptide) was also deleted in the rBCG mutants. **Conclusion:** We have demonstrated that *E.coli-Mycobacteria* expression vectors containing a weak promoter and lysine complementing gene in BCG host strain lysine auxotrophic do prevent genetic rearrangements involving toxic components and protective B-cell and CTL epitopes from HIV-1 envelope. A critical issue for rBCG based HIV-1 vaccine development.

CONFERENCIA INTERNACIONAL SOBRE VACUNAS VIH. Quebec, septiembre 2005

Identification and recruitment of a high risk population for HIV vaccine efficacy trials in Barcelona, Spain. Social and health impact on vulnerable population

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Background: Identification and follow-up of HIV-negative high-risk individuals is necessary for the prevention of HIV transmission and for conducting efficacy HIV-1 preventive vaccine human trials.

Objectives: i)To identify, recruit and follow-up seronegative individuals at high risk of HIV infection; ii) To estimate the HIV, HBV and HCV prevalence; iv) To evaluate the willingness of this cohort to participate in such trials in Spain.

Methods: This multicentric study has involved the Hospital Clínic (HC) and two community based organizations: Red Cross Centre (CR), detoxification Unit for drug users and Ambit Prevenció (AP), day care center for sex-workers on the streets. Epidemiological data, behavior risk factors and willingness were recollected using a confidential questionnaire. HIV positive individuals were referred to a specialized

health center. HIV negative individuals were enrolled in this study and followed-up over 1 year.

Results: We show our preliminary data on 176 subjects (81 CR ,76 from AP and 19 from HC) followed-up for 12 months in CR and , 6 months in HC and AP. There were 88 men, 82 women and 6 transexual. 52% of them were immigrants, 27% were injecting drug users (IDU), 59% others drugs users and 46% were sex-workers on the streets. The overall HIV seroprevalence was 6% (10/176), 5 of them from CR and 5 from AP. All individuals from HC (stable serodiscordant partners) were seronegative. Also, the hepatitis C virus seroprevalence was 50 % (51/103), 94% were IDUs from CR. Seroprevalence of hepatitis B virus (Anti-HBc) was 18 % (19/105). Among seronegative participants 158/166 (95%) affirmed that they would participate in efficacy HIV vaccine trial.

Conclusion: This study provides important data for planning future vaccine efficacy studies in Spain and shows that research involving vulnerable population and interaction between health centers and NGOs is important to implement HIV prevention programs.

Alpha-antigen promoter from *Mycobacteria spp* and lysine gene complementation prevent the disruption of heterologous HIV-1 gp120 gene expression by genetic rearrangements in BCG strain lysine auxotrophic. A critical issue for recombinant BCG based HIV-1 vaccine

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A promising approach for an HIV vaccine is *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) as bacterial live recombinant vaccine vehicle. In this study, we have evaluated the disruption of heterologous HIV-1 gp120 gene expression by genetic rearrangements in BCG host Pasteur strain using a replicative (pMV261) vector. We have also employed the replicative (pJH222) vector in BCG host strain lysine auxotrophic complemented with a plasmid carrying a lysine complementing gene from *M. smegmatis* to compare the HIV-1gp120 gene expression and plasmid stability *in vivo*. The entire DNA coding sequence of HIV-1 gp120 (HXBc2 strain) was amplified by PCR and cloned into different *Escherichia coli- Mycobacteria* shuttle vectors under the regulatory control of BCG *hsp60* promoter (pMV261) and *Mycobacteria* spp. alpha-antigen promoter (pJH222). 12 out of 14 rBCG:HIV-1gp120(261) colonies screened showed a partial deletion in the DNA coding sequence and 2 did show a total deletion. On the other hand, 10 out of 10 rBCG:HIV-1gp120(222) colonies screened kept the fragment DNA intact. Strikingly, the DNA sequence coding for HIV-1 envelope immunodominant CTL epitope (P18 peptide) was also deleted in the rBCG mutants. We have demonstrated that *E.coli-Mycobacteria* expression vectors containing a weak promoter and lysine complementing gene in BCG host strain lysine auxotrophic do prevent genetic rearrangements involving toxic components and protective B-cell and CTL epitopes from HIV-1 envelope. A critical issue for rBCG based HIV-1 vaccine.

1. Introduction

The need for a safe and effective HIV vaccine has never been greater. The UNAIDS report on the global AIDS epidemic updated on december 2004 states that the total number of people living with the human immunodeficiency virus (HIV) has reached its highest level ever: an estimated 39,4 million people are living with HIV and 4,9 million people became newly infected with HIV in 2004. In the past year , the global AIDS epidemic killed 3,1 million people, and by 2010, the number of children orphaned by AIDS could be around 25 million [1]. It is estimated that 85% of these new infections occur in developing countries. In addition, ensuring universal access to antiretrovirals in developing countries still presents an enormous challenge [2].

There is strong evidence supporting a role of cytotoxic T lymphocytes (CTLs) in the containment of HIV replication. Several vaccine approaches are being taken to elicit anti-HIV CTL responses. A promising approach is *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) as bacterial live recombinant vaccine vehicle. BCG vaccine has been used to immunize more than two billion individuals against tuberculosis with a long record of safe use in humans and is able also to induce long lasting immunity. CTL induction against HIV-1 and SIV gag or env antigens has been described following immunization of mice or rhesus monkeys with recombinant BCG (rBCG) expressing these antigens.

The development and understanding of mycobacterial genetic system has been hindered by the slow growth and special cell wall characteristics of the organism like tendency to aggregate. Many important contributions have provided understanding of this genetic system. Bloom et al. (1987) initially reported the development of E.coli-mycobacterial shuttle plasmids(8). They identified a 1.8-kilobase segment (oriM) from plasmid pAL5000 isolated from *Mycobacterium fortuitum,* which supports plasmid replication in M. Smegmatis and BCG. Yamada´s group established a foreign antigen secretion system in mycobacteria using α-antigen promoter to express foreign antigens (9). Stover and collegues developed two different systems for propagating foreign DNA in mycobacteria: a multicopy extrachromosomal plasmid and single-copy plasmid, which is integrated into mycobacterial chromosome (10;11). Important contribution has been done by Tyagi et al (2000) on rBCG approach for development of vaccines. They have

developed a generic vector system for expression of genes at varying levels in mycobacteria under the control of a battery of mycobacterial promoters of varying strength for elicitation of optimal immune responses(12).

Critical issues to be considered in developing rBCG technology include: i) antigen localization;ii) codon optimization;iii) plasmid stability and iv) genetic rearrangements. Genetic rearrangements have been described and occur in eukaryotes and prokaryotes. They may create new sequences, alter functions of existing sequences or place them in new regulatory situations. Have been reported that mycobacteria contains several insertion sequences (IS), and have been described that some of them possess transposition activity in Mycobacterium smegmatis and BCG. The role of such elements has not been explored in heterologous gene expression in mycobacteria. In this study we have evaluated the disruption of heterologous HIV-1 gp120 (HXBc2 strain) gene expression by genetic rearrangements in *Mycobacterium Bovis* BCG host strain using a replicative (pMV261::HIV-1gp120) vector . We have also employed the replicative (pJH222::HIV-1gp120) and integrative (pJH223::HIV-1gp120) vectors in lysine auxotrophic BCG host strain complemented with a plasmid carrying a wild-type complementing gene, ensuring the maintenance of the recombinant antigen gene to compare the HIV-1gp120 gene expression and plasmid stability *in vivo*. In case of replicative pMV261::HIV-1gp120 vector, loss of HIV-1gp120 expression was due to a consensus DNA fragment deletion in the HIV-1gp120 gene detected in 10 different BCG clones by PCR. We have not detected any loss of HIV-1gp120 expression in the replicative (pJH222:HIV-1gp120) and integrative (pJH223:HIV-1gp120) vectors.

2. Materials and methods

2.1.Bacterial strains and culture methods

The bacterial strains used in this study are listed in Table 1. *Escheriachia coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar plates at 37^0C. LB was supplemented with kanamycin (40 μg/ml). BCG wild type and Lysine auxotroph of BCG strains were transformed by electroporation. Mycobacterial cultures were grown in Middlebrook 7H9 broth (Difco) or on Middlebrook agar 7H10 medium (Difco) supplemented wit albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and kanamycin (25 μg/ml). The L-Lysine Monohydrochloride was obtained

from Sigma Chemical, dissolved in destilled water, and used at a concentration of 40 μg/ml.

2.2 Electroporation of mycobacteria

For transformation , BCG cultures were grown to an O.D of 0.9 (600nm), sedimented at 3000 rpm , and washed twice by resuspension and centrifugation (3000 rpm) in 10 % glycerol at 4^0 C, and finally resuspended in 1/20th of the original culture volume of cold 10 % glycerol. Then 100 μl of the cold BCG suspension was mixed with plasmid DNA (50-500ng) in a prechilled 0.2 cm electroporation cuvette and transformed using the Biorad Gene Pulser electroporator at 2.5kV, 25 mf, and 1000Ω. After electroporation 1mL 7H9 medium supplemented with ADC and containing Tween 80 and incubated at 37^0C for 12 hours before plating on Middlebrook agar 7H10 medium (Difco) supplemented with albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and kanamycin (25 μg/ml).

2.3.Construction of Expression vectors

The plasmids and the E.coli / mycobacterial shuttle vectors used to express HIV-1 gp120 (SHIV-HXBc2P 3.2) antigen are listed in Table 1 and 2. Plasmid vectors pMV261 , pJH222 and pJH223 were used as parental plasmids for all the plasmid constructs described below. Plasmid pMV261 and pJH222 are replicative vectors and pJH223 is an integrative vector. All of them contain a DNA cassette encoding kanamycin resistance , an E.coli origin of replication (*oriE*), and an expression cassette containing a mycobacterial promoter, a multiple cloning site and a transcriptional terminator. The pMV 261 and pJH222 contains a mycobacterial plasmid origin of replication (*oriM*) and the pJH223 plasmid contains a DNA segment carrying the attachment site (*attP*) and the integrase (*int*) gene from the mycobacteriophage L5. The entire DNA coding sequence of HIV-1 gp120 was synthesized by PCR , using oligonucleotide primers specific for HIV-1 gp120 gene and cloned into different *Escherichia Coli- Mycobacterial* shuttle vectors under the regulatory control of BCG *hsp60* promoter (pMV261) and *Mycobacteria spp*. alpha-antigen promoter (pJH222 and pJH223) . The coding sequence was fused to *Mycobacterium tuberculosis* 19kD lipoprotein signal sequence (pJH222 and pJH223). For immunodetection purpose we fused downstream of the HIV-1 gene the influenza peptide (hemagglutinin epitope) coding sequence and six residues of histidine. The oligonucleotides were designed to

incorporate BamHI and HindIII sites at the 5' and 3 ' termini of the amplified DNA fragment in pMV261 plasmid, and HindIII-HindIII in pJH222 and pJH223 plasmids.

2.4. Plasmid DNA isolation and DNA sequencing

The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used for isolation of plasmid DNA from mycobacteria and from E.coli. For mycobacteria we used the standard QIAprep Spin Miniprep protocol with one change. After addition of buffer P1, lysozyme was added at a concentration of 10 mg/ml and samples were incubated at 37^0 C overnight. The standard protocol was then followed. The mycobacterial plasmid DNA was transformed in E.coli JM109 using 5ul of the isolated plasmid DNA and purified according to the manufacturer's instructions and finally the plasmids pMV261:: HIVgp120 were used as templates for DNA sequencing and for PCR analysis. The nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (applied Biosystems) and ABI PRISM™ 3100 Genetic Analyzer. DNA sequence alignments were performed using the clustal software.

2.5. Western blot analysis

BCG transformants were grown to mid-logarithmic phase in liquid 7H9 (Difco) medium containing kanamycin (25 μg/mL). rBCG cultures were centrifugued at 3000 rpm for 10 minutes at 4^0C. Pellets were washed twice in PBS plus 0.02 % Tween-80 and resuspended in 1ml of extraction buffer (50mM Tris-HCl pH 7.5, 5mM EDTA, 0.6 % sodium dodecyl sulfate) and 5 μl of 100x protease inhibitor cocktail (1mg/ml aprotinin, 1mg/ml E-64, 1mg/ml leupeptin, 1 mg/ml pepstatin A, 50mg/ml pefabloc SC, and 10 ml DMSO) was added. Cells were sonicated for 4 minutes on ice on Branson sonifier at output control 7, duty cycle 50%. Extracts were centrifugued at 13000 rpm for 10 minutes at 4^0C and supernatants were collected. Proteins were separated on 15 % SDS-polyacrylamide gel . After electroblotting , nitrocellulose membranes were first probed with mouse monoclonal antibody HA.11 directed to influenza hemagglutinin epitope (YPYDVPDYA) and second with HRP-conjugated antibodies.

3. Results

3.1 Cloning of HIV-1gp120 gene into different E.coli-mycobacterial expression vectors

The cloning of the HIV-1gp120 gene was confirmed by enzyme restriction analysis.

3.2 DNA fragment deletion detected by PCR and confirmation by restriction enzyme analysis

As shown the figure ,we detected by BCG colonies PCR the partial deletion using the specific primers that we used for cloning the DNA sequence corresponding to HIV-1gp120 gene into pMV261 vector. This genetic rearrengement was detected and reproduced also in 12 out of 14 rBCG:HIV-1gp120 (261) colonies by PCR (data not shown). As we can observe in figure , this partial deletion was observed only by BCG colonies PCR when the DNA fragment was inserted into pMV261 vector that contains the hsp60 promoter from BCG. We did not detected by BCG colonies PCR the deletion when the DNA fragment was cloned into pJH222 and pJH223 vectors that contain the alpha antigen promoter. We also did compare the restricton enzyme digestion profile of the plasmid DNA vector pMV261 with and without heterologous DNA insert and pJH222 containing the heterologous DNA insert, pre and post BCG transformation. As shown in figure , the pMV261 plasmid vector without the DNA insert kept the same profile pre and post BCG transformation. Contarily, the pMV261 containing the DNA insert did show a different restriction profile pre and post BCG transformation. On the other hand, the pJH222 plasmid vector containing the DNA insert kept also the same restriction profile pre and post BCG transformation

3.3 Nucleotide sequence analysis and characterization of the HIV-1gp120 gene

12 out of 14 rBCG:HIV-1gp120(261) colonies screened showed a partial deletion in the DNA coding sequence and 2 did show a total deletion. On the other hand, 10 out of 10 rBCG:HIV-1gp120(222) colonies screened kept the fragment DNA intact (DNA sequencing confirmation). The partial deletion (900 bp) corresponds to gp120 core containing 3 glycosilations sites and V3, V4 and V5 regions of HIV-1 env. All rBCG mutants kept the ORF and one of them was analyzed by Western blot detecting a band of 25-KDa (truncated protein) and the expected 67-KDa of the rBCG:HIV-1gp120(222) recombinant protein. Strikingly, the DNA sequence coding for HIV-1 envelope immunodominant CTL epitope (P18 peptide) was also deleted in the rBCG mutants. No deletions were observed with pJH222 and pJH223 plasmids, which contains alpha

antigen promoter, indicating that the deletions were not due solely to lethal effects of expression of HIV-1gp120 during growth of the recombinant BCG strains. In addition, we did confirm by enzyme restriction analysis that plasmid pMV261, which contains the hsp60 promoter without an attached expressed gene, did not exhibit deletions after BCG transformation.

3.4 Expression of HIV-1gp120 in rBCG

Expression of HIV-1gp120 protein from all the rBCG expression vectors was confirmed by SDS-PAGE and western Blot analysis of whole-cell BCG lysates. A HIV-1gp120 gene encoding gp120 protein from SHIV-HXBc2P 3.2 was cloned into rBCG expresion vectors pMV261, pJH222 and pJH223 to result in vectors pMV261::HIVgp120, pJH222::HIVgp120 and pJH223::HIVgp120. For pMV261::HIV gp120 the HIVgp120 gene was fused to the first six codons of the cytoplasmically expressed BCG hsp60 gene. For the pJH222::HIVgp120 and pJH223::HIVgp120 the HIVgp120 gene was fused to the 5' region of the Mtb 19 gene encoding the lipoprotein signal peptide for the *M Tuberculosis* Mtb19 surface lipoprotein.

In pMV261 vector expression of the HIVgp120 gene was driven by the BCG *hsp60* promoter on a multicopy extrachromosomal plasmid vector. In pJH222 and pJH223 vector expression of the HIVgp120 gene was driven by *Mycobacterium spp.* alpha-antigen promoter on a multicopy extrachromosomal plasmid vector and monocopy and integrative repectively. The chimeric 19KD lipoprotein-HIV-1gp120-Flu-His protein (relative molecular mass 67082; Mr 67.08 K) was present in lysates of BCG cells containing the 1.82 kilobase (kb) DNA coding sequence. The apparent Mr of HIV-1gp120 protein was consistent with that predicted by the gene sequence in the absence of post-translation modification. Proteolytic fragments of the HIV-1gp120 protein were also detected

4. Discussion

Deepak Kumar et al. did study the stability of the expresssion of B-galactosidase in Mycobacterium smegmatis using integrative and repliactive vectors. In case of replicative vectors, they detected deletions of different sizes in the lacZ gene. Genetic rearrangements have been described and occur in eukaryotes and prokaryotes. They

may create new sequences,alter functions of existing sequences or place them in new regulatory situations. We have demonstrated that weak promoters and BCG lysine auxotrophic host strain complemented with lysine gene do prevent this genetic rearrangements. The results obtained show that such rearrangements can contribute significantly to the expression of foreign genes in mycobacteria. This is a critical issue to be considered for genetic engineering of BCG based HIV vaccine.

Table 1. List of bacterial strains and plasmids and used in this study

Strains and plasmids	Relevant characteristics	Reference or source
Bacterial strains		
Mycobacteria bovis BCG	1173 P2 Pasteur strain	
Mycobacteria bovis BCG mc^2 1604	Pasteur ΔlysA5::res	JBact 1999 4780-4789 Pavelka+jacobs
E.coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(rK$^-$mk$^+$), e14$^-$(*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lac I* ^{q}, *lacZ*ΔM15]	GIBCO BRL
Plasmids		
pMV261	Km^r; E.coli-mycobacterial shuttle vector; Replicative and extrachromosomal vector.	Stover et al.1991Nature351:456-460
pJH222	Replicative extrachromosomal vector, kanamycin resistant, lysine A complementing gene. pMV261 derivative	Barry Bloom and W Jacobs laboratory
PJH223	Integrative vector, kanamycin	Barry Bloom and W

	resistant,lysine A complementing gene. pMV306 derivative	Jacobs laboratory
pMV261::HIV-1gp120		This work
pJH222::HIV-1gp120		This work
PJH223::HIV-1gp120		This work

Table 2. BCG/E.Coli shuttle expression vectors used to express HIV-1gp120

Plasmid vector	**PROMOTER**	**SECRETION SIGNAL**
pMV261::HIV-1 gp120	BCG hsp60	None
pJH222::HIV-1 gp120	α-antigen of Mycobacterium spp	exported mycobacterial lipoprotein
pJH223::HIV-1 gp120	α-antigen of Mycobacterium spp	exported mycobacterial lipoprotein

Cloning and expression of HIVA gene in lysine auxotrophic *Mycobacterium bovis* BCG strain. Enhanced specific HIV immune responses using BCGHIVA prime/ MVAHIVA boost in Balb/c mice immunization.

Elias Pezzat et al.

1. Introduction

The need for a safe and effective HIV vaccine has never been greater. According to 2004 Report from UNAIDS on the global AIDS epidemic, the number of people living with HIV continues to grow- from 35 million in 2001 to 38 million in 2003 and almost five million people became newly infected with HIV in 2003. In the same year, almost 3 million people were killed by AIDS , and by 2010, the number of children orphaned by AIDS could be around 25 million.

There is strong evidence supporting a role of cytotoxic T lymphocytes (CTLs) in the containment of HIV replication. Several vaccine approaches are being taken to elicit anti-HIV CTL responses. A promising approach is *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) as bacterial live recombinant vaccine vehicle. BCG vaccine has been used to immunize more than two billion individuals against tuberculosis with a long record of safe use in humans and is able also to induce long lasting immunity. CTL induction against HIV-1 and SIV gag or env antigens has been described following immunization of mice or rhesus monkeys with recombinant BCG (rBCG) expressing these antigens.

The development and understanding of mycobacterial genetic system has been hindered by the slow growth and special cell wall characteristics of the organism like tendency to aggregate. Many important contributions have provided understanding of this genetic system. Bloom et al. (1987) initially reported the development of E.coli-mycobacterial shuttle plasmids(8). They identified a 1.8-kilobase segment (oriM) from plasmid pAL5000 isolated from *Mycobacterium fortuitum,* which supports plasmid replication

in M. Smegmatis and BCG. Yamada´s group established a foreign antigen secretion system in mycobacteria using α-antigen promoter to express foreign antigens (9). Stover and collegues developed two different systems for propagating foreign DNA in mycobacteria: a multicopy extrachromosomal plasmid and single-copy plasmid, which is integrated into mycobacterial chromosome (10;11). Important recent contribution has been done by Tyagi et al (2000) on rBCG approach for development of vaccines. They have developed a generic vector system for expression of genes at varying levels in mycobacteria under the control of a battery of mycobacterial promoters of varying strength for elicitation of optimal immune responses(12).

Previously, Hanke and McMichael 2000, had constructed a DNA prime-MVA boost candidate HIV vaccine expressing a common immunogen, designated HIVA. It is derived from consensus HIV-1 clade A gag p24/p17 sequences and a string of clade A CTL epitopes, it does not contain the envelope gene and focuses solely on the induction of cell-mediated immune responses. In pre-clinical studies, both the pTHr.HIVA DNA and MVA.HIVA vaccine components were highly immunogenic in mice and macaques (Hanke and McMichael 2000; Wee et al. 2002).

In this study, we have firstly cloned the HIVA gene in lysine auxotrophic *Mycobacterium Bovis* BCG host strain using a replicative (pJH222::HIVA) vector and integrative (pJH223::HIVA) vector. Both plasmids were carrying the wild-type lysine complementing gene, to ensure the maintenance of the recombinant antigen. Secondly we have evaluated the HIVA protein expression by the recombinant BCG strains and finally, we have studied in a murine model the specific HIV immune responses against HIV-1 envelope immunodominant CTL epitope (P18 peptide) and against three CD4+ helper T-cell epitopes from HIV-1 gag , p24 region after Balb/c mice immunization using the BCG prime-MVA boost expressing the HIVA immunogen. According our knowledge, this is the first report on rBCG based HIV vaccine using BCG auxotrophic strains in a murine model. The immunogenicity data has been compared with *Mycobacterium Bovis* BCG Pasteur host strain using a replicative (pMV261::HIVA and pMV261::GFPHIVA) vector.

2. Materials and methods

Bacterial strains and culture methods

The bacterial strains used in this study are listed in Table 1. Escheriachia coli cultures were grown in Luria-Bertani (LB) broth or on LB agar (Difco). LB was supplemented with kanamycin (40 μg/ml). BCG wild type and Lysine auxotroph of BCG strains were kindly provided by W.R. Jacobs and B.Bloom and transformed by electroporation. Mycobacterial cultures were grown in Middlebrook 7H9 broth (Difco) or on Middlebrook agar 7H10 medium (Difco) supplemented with albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and kanamycin (25 μg/ml). The L-Lysine Monohydrochloride was obtained from Sigma Chemical, dissolved in water, and used at a concentration of 40 μg/ml.

Electroporation of mycobacteria

For transformation , BCG cultures were grown to an O.D of 0.9, sedimented at 3000 rpm , and washed twice by resuspension and centrifugation (3000 rpm) in 10 % glycerol at 4^0 C, and finally resuspended in 1/20th of the original culture volume of cold 10 % glycerol. Then 100 μl of the cold BCG suspension was mixed with plasmid DNA (50-500ng) in a prechilled 0.2 cm electroporation cuvette and transformed using the Biorad Gene Pulser electroporator at 2.5kV, 25 mf, and 1000Ω. After electroporation 1mL 7H9 medium supplemented with ADC and containing Tween 80 and incubated at 37C for 12 hours before plating on Middlebrook agar 7H10 medium (Difco) supplemented wit albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and kanamycin (25 μg/ml).

Construction of Mycobacteria Expression vectors

The plasmids used in this study are listed in Table 1 and 2. Plasmids , pMV261, pJH222 and pJH223 were kindly provided by W.R. Jacobs and B.Bloom and used as parental plasmids for all the plasmid constructs described below. Plasmid pMV261 and pJH222 are replicative vectors and pJH223 is a integrative vector. All of them contain a DNA cassette encoding kanamycin resistance , an E.coli origin of replication (oriE), and an expression cassette containing a mycobacterial promoter, a multiple cloning site and a transcriptional terminator. The pJH223 contains a mycobacterial plasmid origin of replication (oriM) and a DNA segment carrying the attachment site (attP) and the integrase (int) gene from the mycobacteriophage L5. The DNA coding sequence of

HIVA was synthesized by PCR , using oligonucleotide primers specific for HIVA gene and cloned into different Escherichia Coli- Mycobacterial shuttle vectors under the regulatory control of BCG hsp60 promoter (pMV261) and Mycobacteria spp. alpha-antigen promoter (pJH222 and pJH223) . The coding sequence was fused to Mycobacterium tuberculosis 19kD lipoprotein signal sequence (pJH222 and pJH223). The oligonucleotides were designed to incorporate BamHI and HindIII sites at the 5' and 3 ' termini of the amplified DNA fragment in pMV261 plasmid, and HindIII-HindIII in pJH222 and pJH223 plasmids.

In vivo stability of plasmid rBCGHIVA.

The growth of rBCG and the stability of the extrachromosomal plasmid (pMV261::HIVA and pJH222::HIVA) were established by the recovery of rBCG from spleens of mice 15 days after immunization . Spleens were homogenized and plated on Middlebrook 7H10 medium supplemented with kanamycin (25 μg/mL). The DNA fragment coding for HIVA was amplified by BCG colonies PCR using specific primers. For ensuring the maintenance of the recombinant antigen gene, the plasmid did containg the complementing Mycobacteria Tuberculosis lysine A gene

Western blot analysis

BCG transformants were grown to mid-logarithmic phase in liquid 7H9 (Difco) medium containing kanamycin (25 μg/mL). rBCG cultures were centrifugued at 3000 rpm for 10 minutes at 4^0C. Pellets were washed twice in PBS plus 0.02 % Tween-80 and resuspended in 1ml of extraction buffer (50mM Tris-HCl pH 7.5, 5mM EDTA, 0.6 % sodium dodecyl sulfate) and 5 μl of 100x protease inhibitor cocktail (1mg/ml aprotinin, 1mg/ml E-64, 1mg/ml leupeptin, 1 mg/ml pepstatin A, 50mg/ml pefabloc SC, and 10 ml DMSO) was added. Cells were sonicated for 4 minutes on ice on Branson sonifier at output control 7, duty cycle 50%. Extracts were centrifugued at 13000 rpm for 10 minutes at 4^0C and supernatants were collected. Proteins were separated on 15 % SDS-polyacrylamide gel . After electroblotting , nitrocellulose membranes were first probed with anti-Pk mAb and second with HRP-conjugated antibodies.

Mouse immunizations and isolation of splenocytes. Groups of 5-6 week-old female BALB/c mice will be injected s.c with an indicated number of 10^7 cfu of rBCG or into

their anterior tibial muscles (i.m.) with 10^6 pfu of rMVA under general anaesthesia. At various times after the last immunization, the animals will be sacrificed, their spleens will be removed and pressed individually through a cell strainer (Falcon) using a 2-ml syringe rubber plunger. The splenocytes will be washed twice and suspended in 10 ml of Lymphocyte medium (RPMI 1640 supplemented with 10% FCS penicillin/ streptomycin, 20 mM HEPES and 15 mM 2-mercaptoethanol). All animal procedures and care strictly conformed to the Spanish and U.K. Home Office Guidelines.

Production of tetrameric MHC/peptide complexes. The genes coding for the Mamu-A*01, and H-2D^d chains will be kindly provided by Drs DI Watkins (Wisconsin Regional Primate Research Center, USA) and JD Altman (Emory University School of Medicine, USA). Tetrameric complexes will be prepared using standard procedures (Hanke et al., 1999). Briefly, both heavy and light chains of MHC will be expressed in E. coli strain BL-21, purified from inclusion bodies, denatured in 8M urea and refolded in the presence of peptide, biotinylated using the BirA enzyme (Avidity) and purified on FPLC and monoQ ion exchange columns. The formation of tetrameric complexes will be induced by addition of chromogen-conjugated streptavidin (ExtrAvidin; Sigma) to the refolded biotinylated monomers at molar ratio of MHC-peptide monomer:PE-streptavidin of 4:1. Labeled tetrameric complexes will be stored in the dark at 4 ^{o}C until use.

Mouse lymphokine ELISPOT assay. The ELISPOT assay will be carried out using the Mouse Lymhokine Secreting Cell Kit (BD Biosciences, UK) according to the manufacturers instructions. In brief, 10^5 isolated splenocytes depleted of red blood cells will be restimulated in duplicates in anti-lymhokine-precoated 96-well plates with R10 alone, supplemented with concanavalin A at 4 □g/ml or specific peptide at 2 □g/ml for 18 hours at 37 □C in 5% CO_2. Following lysis of the cells by a 10-minute incubation with water on ice, spots will be visualised using sequential applications of a biotin-conjugated secondary anti-lymhokine antibody, avidin-horseradish peroxidase and AEC (3-amino-9-ethyl-carbazole, Sigma, UK) and H_2O_2(30%). Spots will be counted using an ELISPOT reader (Autoimmun Diagnostika GmbH, Germany) and expressed as spot-forming units per 10^6 splenocytes.

Mouse intracellular cytokine staining. Isolated mouse splenocytes will be stimulated with appropriate peptide- or peptide pool-pulsed P815 cells in the presence of anti-

CD28/antiCD49d mAbs for 90 minutes at 37 □C in 5% CO_2. Brefeldin A will be then added to inhibit cytokine secretion and the samples will be incubated for additional 6 hours before terminating the reaction with EDTA and the FACS fix solution. The cells will be permeabilized and incubated with anti-CD8-PE (BD PharMingen) and anti-lymhokine-FITC (BD PharMingen) monoclonal antibodies and analyzed using flow cytometry.

3. Results

Cloning of HIA gene into different E.coli-mycobacterial expression vectors

The cloning of the HIVA gene was confirmed by restriction analysis.

Expression of HIVA in rBCG

Expression of HIVA protein from the rBCG expression vectors was confirmed by SDS-PAGE and western Blot analysis of whole-cell BCG lysates. The HIVA gene was cloned into rBCG expresion vectors pMV261, pJH222 and pJH223 to result in vectors pMV261::HIVA, pJH222::HIVA and pJH223::HIVA. For the pJH222::HIVA and pJH223::HIVA the HIVA gene was fused to the 5' region of the Mtb 19 gene encoding the lipoprotein signal peptide for the *M Tuberculosis* Mtb19 surface lipoprotein. For the pMV261::HIVAGFP, the HIVA gene was cloned upstream of GFP DNA coding sequence .

In pMV261 vector expression of the HIVA gene was driven by the BCG *hsp60* promoter on a multicopy extrachromosomal plasmid vector. In pJH222 and pJH223 vector expression of the HIVA gene was driven by *Mycobacterium spp.* Alpha-antigen promoter on a multicopy extrachromosomal and monocopy and integrative respectively. Proteolytic fragments of the HIVA protein were also detected. The expression of the HIV gene segment was driven by BCG hsp60 promoter on a multi-copy extrachromosomal plasmid vector (pMV261) and by Mycobacteria spp. alpha-antigen promoter (pJH222 and pJH223). The chimeric 19KD lipoprotein-HIV-1gp120-Flu-His protein (relative molecular mass 67082; Mr 67.08 K) was present in lysates of BCG cells containing the 1.82 kilobase (kb) DNA coding sequence. The apparent Mr of HIV-1gp120 protein was consistent with that predicted by the gene sequence in the absence of post-translation modification.

4. Discussion

Table 1. List of plasmids and strains used in this study

Strains and plasmids source	Relevant characteristics	Reference or
Bacterial strains		
Mycobacteria bovis BCG	1173 P2 Pasteur strain	
Mycobacteria bovis BCG mc^2 1604	Pasteur ΔlysA5::res	JBact 1999 4780-4789 Pavelka+jacobs
E.coli JM109		GIBCO BRL
Plasmids		
pMV261	Km^r; E.coli-mycobacterial shuttle vector; Replicative and extrachromosomal vector.	Stover et al.1991Nature351:456-460
pJH222	Replicative extrachromosomal vector, kanamycin resistant, lysine A complementing gene. pMV261 derivative	Barry Bloom and W Jacobs lab
PJH223	Integrative vector, kanamycin resistant,lysine A complementing gene. pMV306 derivative	Barry Bloom and W Jacobs lab
pJH222::HIVA		This work
pJH223::HIVA		This work
pMV261::HIVA		This work
pMV261::GFPHIVA		This work

Table 2. BCG/E.coli shuttle expression vectors used to express HIVA

Plasmid vector	**PROMOTER**	**SECRETION SIGNAL**
pJH222::HIVA	α-antigen of Mycobacterium spp	exported mycobacterial lipoprotein
pJH223::HIVA	α-antigen of Mycobacterium spp	exported mycobacterial lipoprotein
pMV261::HIVA	BCG hsp60	None
PMV261::GFPHIVA	BCG hsp60	None