Recombinant *Mycobacterium bovis* BCG as an HIV Vaccine Vector

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Abstract: HIV-1 has resulted in a devastating AIDS pandemic. An effective HIV/AIDS vaccine that can be used to either, prevent HIV infection, control infection or prevent progression of the disease to AIDS is needed. In this review we discuss the use of *Mycobacterium bovis* BCG, the tuberculosis vaccine, as a vaccine vector for an HIV vaccine. Numerous features make BCG an attractive vehicle to deliver HIV antigens. It has a good safety profile, elicits long-lasting cellular immune responses and in addition manufacturing costs are affordable, a necessary consideration for developing countries. In this review we discuss the numerous factors that influence generation of a genetically stable recombinant BCG vaccine for HIV.

Keywords: Recombinant BCG, *Mycobacterium bovis* BCG, HIV, vaccine vector.

INTRODUCTION

Mycobacterium bovis BCG was developed by Léon Charles Albert Calmette and Jean-Marie Camille Guérin, who were working at the Pasteur Institute in 1908, subculturing virulent strains of tubercle bacillus and testing different culture media. They noted that bacilli grown on a glycerin-bile-potato mixture appeared less virulent, and thus changed the course of their research to see if repeated subculturing would produce a strain that was attenuated and thus could be considered for use as a vaccine. By 1919 they had passaged *M. bovis* 198 times resulting in the development of non-virulent bacilli that were unable to cause tuberculosis disease (TB) in research animals. This BCG vaccine was first used in humans in 1921. However, there were many doubts concerning its safety and protective efficacy. This was not helped by a disaster which occurred in 1930 in Lubeck when 240 infants were vaccinated with BCG that was contaminated with a virulent strain resulting in 72 infants developing TB and dying. It was only after World War II that BCG really came into widespread use. BCG is now the world's most widely used vaccine with over 3 billion doses having been administered worldwide.

Numerous features make BCG an attractive delivery vehicle for heterologous antigens. BCG has a proven safety record and is currently the most widely used vaccine: it has been given to billions of people worldwide with a very low incidence of serious complications [1]. The adjuvant effect of BCG has been exploited in experimental vaccines in animals and man allowing the induction of both humoral and cell-mediated immune responses [2]. As a live bacterial vaccine needing limited purification, BCG is cheap and easy to manufacture. Vaccine cost is particularly relevant when considering large-scale vaccination in developing countries. BCG is one of the most heat-stable of the vaccines in use, and does not require an extensive cold chain for maintenance of efficacy [3]. BCG can be administered at or any time after birth and is unaffected by maternal antibodies, with a single dose sensitizing to tuberculoproteins for 5 to 50 years [1]. In the past BCG was also successfully administered orally [4]. A variety of viral, bacterial, parasitic and human antigens have been successfully expressed in BCG, and in experimental models rBCG has elicited protective immunity against measles, papillomavirus, listeriosis, malaria parasites, pneumococcal infection, pertussis, Lyme disease, leishmaniasis and others [5-12].

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BCG AS AN HIV VACCINE VECTOR

There is information from studying long-term non-progressors and HIV-1 infected individuals who are able to control their viral load (elite controllers) that indicates that control of HIV-1 replication is multifactorial and includes host genetic factors [13] as well as immune responses that target Gag [14-16]. There has only been one HIV vaccine trial that has shown moderate protection from HIV infection and the correlates of protection from this trial are still being determined [17]. Most viral vaccines induce neutralizing antibodies but it has not been possible to design an HIV vaccine that induces broadly cross neutralizing antibodies [18]. The other strategy to develop HIV vaccines has been to construct vaccines designed to induce HIV-specific T cell responses that are polyfunctional and of high breadth and magnitude. These vaccines are designed to reduce HIV viraemia which would prolong the time individuals take to progress to AIDS [19,20]. Non-human primate studies have shown that an effective vaccine for HIV should not only induce CD8+ specific T cells [21, 22] but also protect against both early CD4+ T-cell loss and the onset of immune activation [23]. The importance of protection at the portals of HIV entry, that is the anogential tract, is emphasised [24, 25]. Gastrointestinal and vaginal mucosa are the major sites of entry in natural HIV infection. Babies are infected orally *via* breast–milk [26]. The gut-associated lymphoid tissues (GALT), are the primary sites of HIV replication and dissemination [24, 27, 28]. These tissues are also the major

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sites of CD4+ T-cell depletion during the acute stage of infection [24, 29]. In addition, GALT constitute the major reservoirs of HIV replication during both the acute and the chronic stages even in the presence of highly active antiretroviral therapy [24, 27, 28, 30]. There is a large body of evidence that rationalises the development of mucosal HIV vaccines [25, 31] and thus mucosal vaccination is of central importance in assessing the efficacy of HIV vaccines.

Thus ideally a rBCG-vectored HIV vaccine should induce neutralizing antibodies, prime a polyfunctional T cell response of high breadth and magnitude and induce long term memory at the genital, rectal and intestinal mucosa. The vaccine induced T cell response should include CD8+ and CD4+ specific cells. To achieve this numerous factors need to be assessed. These include the dose of BCG, route of vaccination, choice of BCG strain, targeting of the heterologous antigen, choice of expression signals and choice of vector. Finally a suitable animal model is required to investigate all these. These aspects are reviewed in this paper.

Small Animal Models for the Evaluation of rBCG Vaccines

The potential use of BCG as a live bacterial delivery system for HIV immunogens was first demonstrated in a murine system and humoral and cellular immune responses to various HIV antigens expressed in rBCG have since been demonstrated in mice and guinea pigs (Table **1**) [32-35], especially with intravenous, high dose vaccination regimes [32, 36-40]. Oral vaccination of rBCG expressing HIV and SIV Nef resulted in both systemic and mucosal CTL responses [35, 41, 42]. Subsequently, mice were immunised with a pool of rBCGs expressing SIV Gag, Nef and Env; again immunoglobulin and CTL responses were demonstrated [43]. Oral administration of a rBCG expressing SIV genes to mice and guinea pigs has been shown to elicit SIV-specific CTL responses in gut and spleen [44] and IgA antibodies in faeces [43, 45], demonstrating the ability of rBCG to induce local humoral immunity in the intestinal mucosa. It is well accepted that a protective HIV vaccine must elicit a potent and broadly reactive neutralizing antibody response. The potential of rBCG that secretes a chimeric protein containing the V3-neutralizing epitope of HIV-1 to induce HIV-1-specific neutralizing antibodies has been shown in mice and guinea pigs [33, 36, 46]. This group also assessed Gag-specific IgG production in sera and Gag-specific IFN-γ mRNA expression in PBMCs of guinea pigs vaccinated with a (rBCG-SIVgag) vaccine, intradermally and orally during a 3 year period [47, 48]. Similar high levels of Gag-specific serum IgG was generated over the three year period for both routes of inoculation. Both routes of inoculation also elicited Gag-specific IFN-γ responses through-out the three year period. However, responses were higher 1 year after vaccination in intradermally immunized animals. Other groups have been able to generate good HIV or SIV-specific T cell responses in mice utilising a BCG prime and MVA [49] or protein [50] boost.

Non-Human Primate Models for the Evaluation of rBCG Vaccines

Testing of rBCG vaccines in small laboratory animals such as the mouse and guinea pig is an important initial, cost-effective evaluation of immunogenicity. Further immunogenicity testing in non-human primates prior to clinical testing serves to provide information on how the vaccines may perform in humans. Non-human primates have an anatomical and physiological similarity with humans, and importantly their immune system is comparable to that of humans. In this respect, there is high sequence homology between components of the human immune system and that of non-human primates [51-59]. When testing of BCG vaccines is considered in non-human primates, host molecules implicated in mycobacterial infection responses indicate that several of these molecules are present at levels comparable to that in humans making the testing of BCG vaccines in non-human primates relevant [60-63]. Similarities exist between human and non-human primates in the MHC-DR, -DQ and –DP regions [54, 57], and these products are reactive with specific mycobacterial peptides in both species [61]. Group 1 CD1 molecules, involved in the presentation of nonpeptide mycobacterial products to T cells, are present in both human and non-human primates [62,63] unlike the related CD1 molecule in the mouse which does not participate in resistance to *M. tuberculosis* infection [60]. The human T cell antigen receptor repertoire, including γδ T cells, which recognize several mycobacterial ligands [64, 65] are comparable to those of macaques [52, 66]. We have shown that baboons vaccinated with BCG develop immune responses similar to those of humans. A cellular immune response characterized by a PPD-specific delayed hypersensitivity response and BCG-specific IFN-γ production from PBMC, was a consistent finding in Chacma baboons immunized with the Tokyo and Pasteur strains of BCG [67].

To date, a number of studies with SIV and HIV immunogens expressed in *M. bovis* BCG as a vaccine vector have been conducted in non-human primates (Table **1**). Early works by Yasutomi *et al*., showed an immunization schedule with two intradermal inoculations of rBCG expressing SIVmac251gag was capable of generating a MHC class 1-restricted CD8+ T-cell mediated SIV Gag-specific CTL response in *Mamu-A*01* positive rhesus macaques [68]. The CTL response could be enhanced by a single booster inoculation with a SIVmac Gag immunodominant peptide (p11C) formulated with a lipid A-containing liposome in aluminium hydroxide which was given >1 year after the last rBCG inoculation [69]. Although a vigorous p11C-specific CTL response was generated, which was greater than in animals that received p11C peptide-liposome inoculation alone, none of the animals were protected against a pathogenic SIV challenge. Sequencing of the virus recovered from these monkeys showed that the established viruses had no mutations in the p11C region indicating the viraemia was not a result of the p11C-specific CTL escape. Other early studies in non-human primates confirmed the potential of

Table 1. A Summary of SIV and HIV Immunogens Expressed in *Mycobacterium bovis* BCG as Vaccine Vector

HIV/SIV Gene/Antigens Expressed by rBCG	Experimental Design	Animal Model	Key Results	Ref.
HIV-1 Env gp120 expressed under the control of hsp60	Single inoculation with $2x10^6$ CFU BCG containing integrating vector expressing HIV-1 Env gp120 or model antigens (β-galactosidase or fragment C of tetanus toxin)	BALB/C mice	▪ CTL responses (specific lysis) to HIV-1 Env gp120 immunodominant epitope, P18 peptide ▪ Induction of humoral responses to model antigens (*β*-gal and tetanus toxoid) ▪ Induction of cellular immune responses (IFN-γ production) in animals vaccinated with rBCG-*β*-gal, and not with rBCG-gp120	[39]
HIV-1 gag, pol and env expressed separately under the control of hsp70	Single i.d. or i.v. inoculations with $5x10^6$ CFU of rBCG-gag or rBCG-env	BALB/C mice	▪ Induction of humoral responses to HIV-1 proteins in all mice inoculated intravenously ▪ Induction of CTL responses (specific lysis) in mice inoculated with rBCG-gag; CTL activity attributed to CD8+ splenocytes ▪ Induction of cellular immune responses (IFN-γ & IL-2 production) in animals vaccinated with rBCG-gag	[32]
SIVmac251 gag expressed under the control of hsp70	Two i.d. vaccinations with rBCG at 4 sites (dose: 10^8 CFU) 19 weeks apart	*Mamu-A*01+* rhesus macaques	▪ Immunization elicited MHC class 1-restricted CD8+ SIVgag-specific CTL ▪ No anti-SIVmac gag antibody response was generated in immunized animals	[68]
HIV *env* V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence NTRKSIHIGPGRAFYATGS which has a neutralization sequence identical to that of HIV-1MN	Animals were vaccinated once with rBCG by s.c. injection at a dose of 5 mg (~10^8 CFU; guinea pigs) and 0.1 mg (~$3x10^6$; mice).	Guinea pigs and mice	▪ DTH responses to Env V3 and PPD were induced in guinea pigs after immunization with rBCG-Env V3 and following systemic passive transfer of spleen cells obtained from rBCG-Env V3-vaccinated guinea pigs ▪ CTL responses were induced in mice by vaccination with rBCG-Env V3 ▪ Guinea pig immune antisera had ability to (i) neutralize primary HIV-1 isolates and (ii) block HIV infection in SCID mice	[36]
SIVmac251 gag expressed under the control hsp70	Two i.d. vaccinations with rBCG at 4 sites (dose: 10^8 CFU) 19 weeks apart, subsequent boosting (13 months after) with SIVmac Gag peptide p11C (dose: 1 mg; i.m.) formulated with a lipid-A-containing liposome in aluminium hydroxide, and pathogenic SIV challenge	*Mamu-A*01+* rhesus macaques	▪ Vigorous p11C-specific CTL response generated was greater than those generated by a single modality immunization with p11C peptide-liposome inoculation ▪ No protection was afforded by this CTL response against SIV challenge ▪ Recovered virus after challenge had identical SIV gag demonstrating that the CTL response did not select for viral variant that might escape T-cell immune recognition	[69]
SIVmac251 nef expressed under the control of P_{AN} promoter	Oral immunization with rBCG (10^9 CFU) for 5 consecutive days (total dose of $5x10^9$ CFU)	BALB/c mice	▪ rBCG translocated to oropharyngeal mucosa and intestinal epithelium ▪ Strong systemic and mucosal responses were induced ▪ Specific anti-Nef CTLs demonstrated in intraepithelial CD8β+ cells	[41]
SIVmac251*env* gp110 fused to β-lactamase gene and encoding gene under the control of the *pBlaF** promoter.	Immunization *via* subcutaneous inoculation (dose: 10^7 CFU)	BALB/c mice and guinea pigs	▪ Strong CTL responses and humoral immune responses elicited in both mice and guinea pigs immunized by parenteral routes ▪ The anti-gp110 IgGs produced were able to neutralize *in vitro* growth of virulent SIVmac251 field isolates ▪ Guinea pigs immunized by the oral route produced significant levels of anti-gp110 IgAs in the faeces, demonstrating that rBCG is able to induce local humoral immunity in the intestinal mucosa.	[45]

(Table 1) contd…..

HIV/SIV Gene/Antigens Expressed by rBCG	Experimental Design	Animal Model	Key Results	Ref.
SIVmac251 Nef protein, and large fragments of the Env and Gag proteins.	Immunization *via* different mucosal routes (oral, aerogenic, nasal, and rectal)	BALB/c mice	▪ Local, specific IgA, systemic IgG, strong, specific cytotoxic responses of splenocytes against Nef, Env, and Gag was observed whatever the mucosal route of immunization.	[43]
SIVmac251 Gag, Pol, truncated Env and Nef (separately subcloned into shuttle vector under regulatory control of the *hsp70* promoter)	Animals inoculated i.v. with 4 rBCG constructs containing SIV *gag*, *pol*, *env*, and *nef* combined in a single inoculum (dose: 5-7x10^8 CFU)	Rhesus macaques	▪ Vaccination elicited SIV-specific IgA and IgG antibody, and weak cellular immune responses, including CTL and helper T cell proliferation to Gag, Pol and Env.	[70]
HIV *env* V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	Animals inoculated with rBCG either by nasal (10 μg, ~10^6 CFU), oral (100 μg) or s.c. (100 μg) routes, once a week for 3 consecutive weeks	C57BL/6J mice	▪ High titres of Env V3-specific IgG antibodies were generated in the serum of nasally-immunized animal, maintained for >12 months, and had ability to neutralize clinical HIV-1 isolates *in vitro* ▪ Env V3-specific IgG-producing cells were detected in mononuclear cells obtained from spleen, nasal cavity and salivary gland	[46]
HIV *env* V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	rBCG-HIVEnv inoculated *via* either a single (i.d., i.n. and i.r.) or combination (i.r./i.d. and i.r./s.c.) routes. (i.r. = 80 mg of vaccine once a week for 2-4 consecutive weeks; s.c. and i.d. = 1.0 and 0.1 mg once respectively; i.n. = 10 μg 4 times weekly for 3 weeks)	Guinea pigs	▪ DTH responses to both PPD and Env-V3 peptide were detected in animals inoculated with a combination of i.r. and i.d. routes ▪ Animals inoculated by combined rotes had significantly higher titres of HIV-1-specific IgG and IgA in serum with enhanced neutralization activity ▪ Induction of high levels of IFN-γ and IL-2 mRNA in PBMC, splenocytes and intraepithelial lymphocytes up to 2 years after combination inoculation	[147]
HIV *env* V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	rBCG-HIVEnv inoculated *via* oral route (80 mg of freeze-dried vaccine once a week for 4 consecutive weeks, providing a total dose of 320 mg)	Guinea pigs	▪ Env-V3 peptide-specific DTH responses were detected in immunized animals 1.5 years post inoculation ▪ Env-V3 peptide-specific proliferative responses detected in PBMC and intestinal intraepithelial lymphocytes indicating induction of functional T cells to HIV-1 Env in both systemic and mucosal compartments	[182]
SIVmac251 gag, nef and env (truncated) expressed individually in rBCG	Single i.d. inoculation (5×10^8 CFU) into 5 sites on the back, and rectal or oral boosting doses (6×10^{10} CFU) were administered intragastrically or intrarectally every 2 days for 6 days, (total dose of 18×10^{10} CFU). All the animals were challenged intrarectally with pathogenic SIVmac251	Cynomolgus macaques (*M. fascicularis*)	▪ After i.d. inoculation, monkeys exhibited CTL responses targeted against the three antigens and IFN-γ secretion was observed ▪ A rectal or oral boosting dose elicited anti-SIV IgAs in the rectum of vaccinated monkeys and increased IFN-γ secretion by circulating blood cells. No antibodies were detected and no CD4+ T-cell-mediated anti-SIV responses before challenge were observed ▪ No protection from SIVmac251 challenge	[71]
HIV *env* V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus) sequence	One subcutaneous immunisation of 10mg rBCG env V3 and challenge from week 24 with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-98.6PD at different intervals.	Rhesus macaques	▪ Significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific- neutralized primary HIV-1 isolates *in vitro* ▪ Neutralization was not observed against HIV-1SF33/X4 or primary HIV-1 R5 isolates ▪ Viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was decreased after high-dose SHIV-MN challenge. Replication of pathogenic SHIV-89.6PD was not affected.	[72]

(Table 1) contd…..

HIV/SIV Gene/Antigens Expressed by rBCG	Experimental Design	Animal Model	Key Results	Ref.
SIVmac239 full length *gag* in rBCG-Tokyo downstream of hsp60 promoter	Animals were vaccinated with rBCG *via* i.d. by single inoculation (10 mg) and a replication-deficient vaccinia virus strain (DIs) expressing SIVmac239 *gag-pol* (rDIs-SIVgag) i.v. (10^6 PFU) in heterologous prime-boost or reverse combination. Control groups received combinations containing rBCG with empty plasmid or DIs encoding β-galactosidase. All animals were challenged *via* intra-rectal inoculation with pathogenic 2000 $TCID_{50}$ of SHIV KS661c, a derivative of SHIV89.6	Cynomolgus macaques (*Macaca fascicularis*)	▪ rBCG-SIVgag/rDIs-SIVgag regimen induced high levels of SIV-specific IFN-γ spot-forming cells which afforded effective protective immunity against mucosal challenge with SHIV KS661c ▪ Other vaccination regimens including the opposite combination or the single-modality combinations did not effectively protect against viral challenge.	[73]
HIV-1 clade A consensus *gag* (HIVA; containing a string of CD8+ T-cell epitopes) fused to *M.tb* 19-kDa signal sequence and expressed in a lysine auxotrophic strain of BCG	rBCG vaccination *via* i.p. (10^6 CFU) Animals boosted with recombinant rMVA expressing HIVA (10^6 PFU)	BALB/c Mice	▪ rBCG prime-vaccination enhanced CD8+ T-cell responses induced by rMVA boost immunization ▪ Heterologous rBCG/rMVA regimen enhanced the vigour and quality of CD8+ T-cell response including a that to a subdominant epitope ▪ This enhancement was directly related to dose, with higher doses effecting greater enhancement ▪ A DNA prime-rBCG boost regimen afforded protection against a surrogate virus challenge	[91]
HIV-1 group M consensus envelope (CON6) either as a surface, intracellular, or secreted protein.	rBCG vaccination *via* i.p. (either 10^8, 10^7, or 10^6 CFU) Animals boosted with recombinant CON6 gp140 protein foruml=ulated in RiBi adjuvant *via* i.p. (50 μg) Mice immunised with rAd5 and rVV in prime/boost combination served as positive controls	BALB/c Mice	▪ rBCG-induced T-cell responses to HIV-1 envelope in spleen were lower than those induced by rAd/rVV prime/boost ▪ rBCG induced comparable responses to rAd-rVV immunization in the female reproductive tract and lungs ▪ T-cell responses induced by rBCG were primarily CD4(+), although rBCG alone did not induce anti-HIV-1 antibody ▪ However, rBCG could prime for a protein boost by HIV-1 envelope protein. Thus, rBCG can serve as a vector for induction of anti-HIV-1 consensus Env cellular responses at mucosal sites	[50]
Full-length SIV Gag protein under the control of *hsp60* promoter (rBCG-SIVGag) 0.5ng/mg	rBCG-SIVGag inoculated i.d by single inoculation of 0.1 mg or oral route (80 mg once a week for 2 consecutive weeks, providing a total dose of 160 mg)	Guinea pigs	▪ IgG2 levels greater than IgG1 ▪ IFN-γ mRNA expression detected in PBMC for both i.d. and oral vaccine groups ▪ i.d. rBCG induced DTH responses to PPD and SIV Gag p27 protein which were maintained for up to 50 weeks ▪ Oral rBCG induced a long-lasting DTH response to the SIV Gag p27 protein, but not to PPD	[47, 48]
Full-length HIV-1 subtype C *gag* under the control of *mtrA* or *katG* promoters and 19-kDa localisation signal sequence	rBCG vaccination *via* i.d. (10^8 CFU) four times All animals boosted *via* i.m. with HIV-1 subtype C $Pr55^{gag}$ virus-like particles (Gag VLPs; 11 μg) 1 year after last rBCG vaccination	Chacma baboons (*Papio ursinus*)	▪ Induction of BCG vector-specific T-cell immune responses and recall responses after subsequent rBCG vaccinations ▪ Development of low but detectable T-cell responses to HIV-1 Gag after rBCG vaccination and which were efficiently boosted by Gag VLPs ▪ Generation of IFN-γ-producing CD3+CD8+ T cells after rBCG/Gag VLPs prime/boost vaccination ▪ PBMCs from immunised baboons targeted peptides with documented HIV-1 Gag epitopes	[67]

(Table 1) contd…..

HIV/SIV Gene/Antigens Expressed by rBCG	Experimental Design	Animal Model	Key Results	Ref.
Full-length SIVmac239 *gag* and *pol* and a modified *env* under the control of *M.tb* alpha antigen promoter and 19-kDa localisation signal sequence	rBCG vaccination *via* i.d. (10^6 to 10^9 CFU) or i.v. (10^9, 10^8, 10^7, 10^6 CFU) twice, 23 weeks apart All animals boosted *via* i.m. with rAd5 (10^{10} viral particles) expressing SIV Gag/Pol and Env once 20 weeks after last rBCG vaccination	*Mamu-A*01+* rhesus macaques	▪ Induction of BCG vector-specific T-cell immune responses with second rBCG vaccination showing a robust recall response ▪ Development of low but detectable T-cell responses to SIV Gag and Pol but not Env after rBCG vaccination. ▪ Generation of p11C tetramer-binding and highly polyfunctional CD8+T cells after rBCG/rAd5 prime/boost vaccination	[74]

Abbreviations: Intradermal (i.d.); intranasal (i.n.); intrarectal (i.r.); subcutaneous (s.c.); delayed-type hypersensitivity (DTH); purified protein derivative (PPD); severe combined immunodeficiency (SCID).

rBCG vaccines in generating vaccine-specific CTL and helper T cell proliferation as well as IgA and IgG antibody responses in blood [70] and rectal mucosa [71]. Indeed, Mederle *et al*. showed that a single intradermal inoculation with rBCG expressing the SIV *gag*, *nef* and truncated *env* genes followed by either an intra-rectal or intragastric booster inoculation was effective in generating SIV-specific immune responses in the systemic and local mucosal compartments [71]. Although the immune responses did not protect these monkeys against a highly pathogenic SIV challenge, this was a significant finding in view of the fact that a strong motivation for the development of BCG as a HIV vaccine vehicle is based on its potential/ability to induce immune responses in both systemic and mucosal compartments in mice and guinea pig models [23, 36, 46]. Over 80% of HIV transmissions occur *via* the mucosal surfaces and thus, it is likely that HIV vaccines that elicit effective mucosal immune responses would be most efficacious in preventive control of the disease.

Someya *et al*. examined the ability of rBCG to induce HIV-specific neutralising antibodies in a non-human primate model by vaccinating rhesus macaques with a rBCG expressing an HIV-1 Env V3 antigen and evaluating the protection conferred against a pathogenic virus challenge [72]. Vaccination with rBCG resulted in induction of significant levels of neutralizing antibodies which were capable of neutralizing primary HIV-1 isolates *in vitro*. However, whilst this antibody response was not sufficient to protect against an intravenous pathogenic SHIV challenge, it demonstrated significant reduction in the plasma viral load after a non-pathogenic SHIV challenge. In contrast, a prime-boost vaccination of cynomolgus macaques with a rBCG expressing a full-length SIVmac Gag protein and a non-replicating recombinant vaccinia virus expressing SIVmac Gag-Pol was able to elicit effective protective immunity against mucosal challenge with pathogenic SHIV [73]. These data suggest that the protective capacity of a rBCG-vectored HIV vaccine may be enhanced immensely when administered in a heterologous prime-boost combination with the rBCG as the priming vector. Data from other recent studies support the use of rBCG as the primary vaccine in prime-boost vaccinations for maximum vaccine immunogenicity [74, 75].

The role of CD8+ T-cell responses in containment and suppression of viremia in HIV-1-infected humans and SIV- or SHIV-infected monkeys is well documented [76-80]. The magnitude and functional heterogeneity of such T-cell responses have also been associated with better control of viremia in HIV-1 infections [81, 82] and protection of vaccinated monkeys [83]. Studies in long-term nonprogressors strongly suggest that T cells producing multiple cytokines such as IL-2 and TNF-α, in addition to IFN-γ, are important for effective protection [84, 85]. Thus, it is desirable for a rBCG vaccine to elicit a strong and polyfunctional vaccine-specific CD8+ T cell response. In the non-human primate models, rBCG vaccines have been shown to induce vaccine-specific CTLs [68-71, 84] and high levels of IFN-γ-producing cells [73-75]. In addition, Cayabyab *et al.* reported induction of robust vaccine-elicited polyfunctional CD8+ T-cell responses in monkeys after rBCG/recombinant adenovirus 5 prime/boost vaccination [74]. Using intracellular staining and polychromatic flow cytometry, they found that more than half of the CD8+ T cells were polyfunctional, producing two or three of the cytokines IFN-γ, IL-2 and TNF-α. In addition, they observed that a high proportion of SIV Gag p11C tetramer+ CD8+ T cells expressed the three cytokines and CD28, which is a marker associated with central memory. These studies provide additional evidence for the suitability of rBCG in the development of HIV vaccines.

BCG in Heterologous Prime Boost Regimes

In recent years there has been acceptance that for some vaccines, priming with one vaccine vector and boosting with another expressing the same antigen will give an enhanced cellular immune response [86]. Indeed many of the candidate HIV vaccines in clinical trial are being tested using this approach [87, 88]. One of the most promising rBCG prime heterologous boost combinations was reported by Ami *et al.*, where macaques vaccinated with rBCG-SIV*gag* and boosted with replication-deficient vaccinia virus expressing Gag, elicited effective protective immunity against mucosal challenge with SHIV KS661c, whereas animals receiving the opposite combination or the single-modality vaccines were not effectively protected [73]. In TB vaccine trials, BCG is used to prime an immune response that is boosted by MVA expressing Ag85 [86, 89]. In cattle a combination of BCG prime and MVA85A boost gave a response with greater magnitude and breadth than in the BCG\BCG-vaccinated calves [90]. Our laboratory has shown, using Chacma

baboons, that rBCG expressing Gag effectively primes an immune response which can be boosted by Pr55Gag virus like particles (VLP) [75]. Although rBCG alone induced only weak or undetectable HIV-1 Gag-specific responses, the rBCG efficiently primed for a Gag VLP boost and broadened and strengthened the boost. The responses were predominantly CD8+ T cell-mediated and recognized similar epitopes to those targeted by humans with early HIV-1 subtype C infections. In both cases the combination induces a better immune response than either vaccine used individually. Similarly, mice primed with rBCG expressing a string of four $CD8^+$ T-cell epitopes (HIVA) and boosted with rMVA. HIVA generated higher, better quality $CD8^+$ T-cell responses than those immunized with either vaccine alone [91]. Cayabyab and colleagues also showed that monkeys primed with rBCG expressing SIV Gag, Pol and Env and boosted with rAd5 expressing the SIV antigens developed cellular responses of greater magnitude and persistence than those vaccinated with rAd5 alone [74].

FACTORS TO CONSIDER WHEN DESIGNING A RBCG VACCINE VECTOR

BCG is a complex organism and so there are a number of factors that can influence the production of heterologous antigens in BCG.

Choice of Shuttle Vector for rBCG

Escherichia coli/mycobacterial shuttle vectors can either be episomal (self-replicating) or integrative. The first episomal, *E. coli*/mycobacterial shuttle vector was designed by Snapper *et al.*, using a mycobacterial plasmid replicon derived from a natural *Mycobacterium fortuitum* plasmid, pAL5000 [92]. Most episomal mycobacterial vectors contain the pAL5000 origin of replication, which allows approximately five copies of plasmid per cell. Recently Bourn *et al.*, utilized random mutagenesis and positive selection to generate a high copy number version of the pAL5000 replicon, which allows approximately 35 copies of plasmid per cell [93]. A second group, were able to increase the copy number ten fold by over-expressing RepB, a protein that has been shown to be essential for plasmid replication [94]. The integration systems of mycobacteriophages have been used to create vectors which enable site-specific integration between the *attP* phage and *attB* mycobacterial attachment sites in the chromosome. These include use of the L1 mycobacteriophage genes in the phAE19 vector series [92], Ms6 in the pEA4 series [95] and the L5 in the pMH94 [96], pMH947 [97] and pMV361 series [39].

As there are more copies of episomal vector per cell, higher levels of recombinant protein can be expressed than from integrative vectors. This is confirmed by studies carried out by Mederle *et al.*, who compared the stability and levels of foreign antigen expression of a synthetic operon containing the SIV-1 Gag and Nef genes in episomal and integrating vectors in BCG [98]. Expression of Gag was eight to ten fold higher in BCG containing the episomal vector (pJNL29) as compared to the integrative vector (pOIP33). However, this study and a number of others have shown that integrative vectors are more stable than episomal vectors [98, 99]. By removing the transcriptional terminators flanking the synthetic operon, Mederle and colleagues constructed a second integrative vector (pOIP4) that was able to stably express HIV antigens at levels comparable to the episomal vector (approximately 1% of total protein). One hundred days post inoculation in mice, the episomal plasmid was only 25% stable where as the integrative plasmids were 85 to 98% stable. Results indicated that a high and persistent level of Gag expression was necessary for detection of memory T cells, as anti-Gag IFN-γ-producing CD4 T cells could only be detected in mice inoculated with rBCG(pOIP4) six weeks post inoculation. However, similar levels of IFN-γ production was detected in the spleens of mice inoculated with either the episomal or the pOIP4 integrative systems following a purified Gag protein boost. The expression levels of Gag in rBCG(pOIP33) was too low to prime an immune response, suggesting that an initial, high level of antigen expression is necessary to prime an immune response.

Promoter Selection for Transgenes

One of the most important mechanisms regulating the expression of protein is the rate of transcription initiation, which is determined by the strength of the promoter. A good understanding of the strength and expression characteristics of the promoters is thus important for the regulation of expression of heterologous antigens in mycobacteria. Numerous promoters have been used successfully to drive heterologous gene expression in mycobacteria. These include: *hsp60* and *hsp70* from BCG [39], the *M. leprae* 18kDa antigen promoter [100,101], the *M. tuberculosis* 19kDa antigen promoter [11], *mtrA* from *M. tuberculosis*, the P_{AN} promoter from *M. paratuberculosis* [102], the α antigen promoter from *M. kansasii* [38], the *M. fortuitum* β-lactamase promoter (P*blaF*) [103], the *M. tuberculosis* Erp antigen promoter (P*erp*) [103], the acetamidase promoter from *M. smegmatis* [104], and the *groES/groEL1* promoter from *Streptomyces albus* [35].

The promoters that are used most frequently, *hsp60* and *hsp70*, are those of the 60kDa and 70kDa heat-shock proteins (Hsps), respectively. Hsps belong to a family of stress proteins that are essential for normal mycobacterial growth, but are up-regulated during conditions of stress, such as those encountered in the intracellular environment [105]. These promoters have, however, been found to express heterologous antigens constitutively, driving foreign protein expression up to 10% of total BCG protein in some instances [39,101]. A number of groups have shown the promoter activity of *hsp60* to be stronger than that of the 18kDa antigen promoter [100, 106, 107]. However, high expression levels of certain foreign proteins can be toxic to the mycobacterial cell and the *hsp60* promoter has been shown to contribute directly to plasmid instability in a number of studies [108-110]. In contrast, the *M. leprae* 18kDa antigen and *M. tuberculosis mtrA* promoters express foreign antigens relatively weakly *in vitro*, however are strongly induced *in vivo* to levels almost as high as those by *hsp60* [101-112] The choice of promoter is thus a balance between high expression, and the effect of the toxicity or metabolic load the heterologous protein imposes on the bacteria.

Regulated expression systems provide an attractive means of minimizing metabolic load and toxicity during *in*

vitro growth of rBCG vaccine stocks. However, few regulated expression systems are available for control of gene expression in mycobacteria. The acetamidase expression system is controlled by two positive regulators (AmiC and AmiD) and one negative regulator (AmiA). Expression is induced by the presence of acetamide, butyramide or short aliphatic amides in the growth media [104, 113, 114]. However, it is only possible to regulate expression of this system *in vitro*. Ehrt *et al.* developed a gene regulation system based on the *E. coli* Tn10-derived tet regulatory system [115]. In the absence of tetracyline or the analogue anhydrous tetracycline, the Tet repressor (TetR) binds tightly to the tet operators (*tetO*), located within a strong mycobacterial promoter, suppressing expression of the downstream gene. When tetracycline is added to the media it enters the cell and binds to TetR, inducing a conformational change that results in dissociation of the TetR from *tetO* allowing expression from the mycobacterial promoter. Neither of these systems are really suitable for use in vaccines as they require acetamide or tetracycline for induction of expression.

More recently extensive mutagenesis studies in *E. coli* on a chimeric TetR (BD) protein have led to the development of a family of modified TetR proteins that have a reverse phenotype (revTetR) [116]. In this reverse system, gene expression is repressed in the presence of tetracycline, and induced upon its removal. It has been applied in the Gram-positive *Bacillus subtilis* with full regulatory range in which a 500 fold expression induction was achieved [117]. This system was utilized to successfully create conditional knockdown mutants in mycobacteria [104, 113, 114, 118, 119]. By structure-based design and codon-usage adaption to mycobacteria, this group have further improved the reverse TetRs enabling up to 50 fold better repression of reporter genes [119,120]. This system would allow the downregulation of HIV antigen expression whilst culturing rBCG vaccine stocks, by growth in the presence of tetracycline, and upregulation of HIV antigen expression *in vivo*, in the absence of tetracycline.

Codon Optimisation of the Transgene

Mycobacteria have a high G+C content (65%) compared to mammalian genes [121]. As most mammalian genes favour a different codon usage it is not surprising that codon optimisation can increase foreign gene expression in rBCG. This is reflected by an increase in immunogenicity of rBCG expressing codon optimised p24 compared to native p24 [122]. However, there are also some examples of genes with low G+C content that are expressed at high levels in BCG, such as *ospA* of *Borrelia burgdorferi* (30% G+C) [11] and *Streptococcus pneumoniae pspA* (38% G+C) [123].

Targeting of Heterologous Antigen

The accumulation of heterologous proteins in the cytoplasm of bacteria may be detrimental to mycobacteria and so a number of studies have made use of leader peptides to target the protein to the mycobacterial membrane or facilitate secretion of the protein. Fusions of the foreign protein with the signal sequences of the BCG-derived α antigen [11], the α antigen of *M. kanasii* [38], the 85A antigen of *M. tuberculosis* [124, 125] and the *M. tuberculosis* Erp antigen have been used to facilitate foreign antigen secretion. The signal sequence of the *M. tuberculosis* 19kDa lipoprotein has been used extensively to direct foreign antigens to the surface of the mycobacterial membrane [11, 67, 74, 91, 126]. Al Zarouni and Dale tested different combinations of promoters (*hsp60*, 18 kDa, 19 kDa & 85A) and post-translation signals (85A or 19 kDa) coupled with a reporter gene [109]. They found that the 85A antigen secretion signal resulted in increased levels of cell associated protein as compared to the 19kDa signal sequence when utilized in combination with the *hsp60*, 18 kDa or 85A promoters. Levels of secreted or membrane –bound protein were not measured in this study. The membrane bound proteins linked to the 19kDa leader have been shown to be more immunogenic than the cytoplasmic targeted antigens [127]. In our laboratory murine rotavirus VP6 linked to the 19kDa leader sequence was more immunogenic and gave better protection from rotavirus challenge than VP6 linked to the alpha antigen leader sequence [99]. It has also been suggested that removal of foreign antigen from the mycobacterial cell by secretion may support higher levels of its production [128]. Improvement in immune responses and protection on linking of foreign antigen to the 19kDa signal sequence has been noted by others working with rBCG [8, 72, 129, 130]. This improvement may be due to earlier encounter of 19kDa linked foreign antigen with the immune system. In our laboratory we have also shown protection from cotton tail rabbit papillomavirus (CRPV) challenge in rabbits vaccinated with BCG expressing CRPV L1 fused to the 19kDa leader under the control of the *mtrA* promoter [6].

Dose of BCG

A Th1 immune response involves CD8+ and CD4+ T lymphocytes and is cellular in nature. It protects against chronic diseases such as intracellular parasitism and cancer, by activating macrophages and facilitating the detection and lysis of infected cells. Thus, ideally an rBCG HIV vaccine vector should generate a predominantly Th1 response.

It has been proposed that the dose of BCG is crucial in determining the Th1/Th2 nature of the immune response in mice. However, the literature on this topic is controversial and does not account necessarily for the fact that the response could also be affected by the infectivity of the rBCG, the growth of the BCG *in vivo* and the *in vivo* stability of the rBCG. Power *et al*. found that mice vaccinated with low doses of BCG showed an almost exclusively Th1 cell-mediated response whereas a higher dose induced a mixed Th1/Th2 cell mediated response and a Th2-mediated humoral response [131].

In a guinea pig model of TB the protective efficacy of both BCG and a recombinant BCG expressing high levels of the 30kDa major secretory protein of *M. tuberculosis* was independent of dose over a range of $10^1 – 10^6$ live organisms [132]. The humoral response to the 30kDa protein was found to be directly proportional to the dose and seemed to correlate with the persistence of the rBCG30. Low doses of rBCG30 were rapidly cleared from animal organs whereas higher doses persisted for several weeks after immunization. In contrast, although the cell-mediated immune response developed more slowly in animals immunized with a lower

dose of vaccine as compared to higher doses, comparable peak responses were observed.

The host responses to three different rBCG over-expressing the 38 kDa antigen (rBCG38T), the 19 kDa antigen (rBCG19T) or ESAT6 (rBCGE6T) were markedly different [133]. Mice vaccinated with rBCG38T exhibited a predominantly Th1 response, to both the BCG sonicate and the 38 kDa antigen, whereas the host response to rBCG19T was predominantly Th2 in response to BCG sonicate and predominantly Th1 to the 19 kDa antigen. The rBCG-expressing ESAT6 induced a mixed Th1/Th2 response towards the ESAT6 protein but a marked bias towards a Th1 phenotype in response to the BCG sonicate. This group also compared the immune responses elicited in mice vaccinated with rBCG overexpressing Ag 85A or Ag 85C [133]. Recombinant BCG overexpressing Ag 85A induced an increased Th1-like response in comparison to the wild type BCG, whereas rBCG overexpressing Ag 85C did not. This data seems to indicate that the type of response is not only dependent on the dose of BCG, but can also be influenced by the type of antigen expressed.

In another experiment BALB/c mice were immunized with three different rBCG constructs expressing differing levels of Ag85A [134]. The authors found that high levels of expression of Ag85A induced a predominantly Th1 response, whereas high to intermediate levels of Ag85A induced a mixed Th1/Th2 response. However, the immune responses against BCG remained Th1.

Im and colleagues found that a high priming dose of rBCG in mice was particularly important for improving the strength and quality of immune response to subdominant T cell epitopes. Whereas the quality of the immune response to an immunodominant epitope was not significantly effected by dose [91].

Route of Immunization

Many studies have tried to compare the response to foreign antigens delivered by rBCG inoculated *via* different routes, however comparisons are difficult as doses used are usually different and after oral or intra-gastric immunization large numbers of BCG are shed in the faeces and thus only small numbers are taken up by intestinal cells [41,46].

The gut-associated lymphoid tissues (GALT), are the primary sites of HIV replication and dissemination [24, 27, 28]. These tissues are also the major sites of CD4+ T-cell depletion during the acute stage of infection [24, 29]. In addition, GALT constitute the major reservoirs of HIV replication during both the acute and the chronic stages [24,27,28,30] even in the presence of highly active antiretroviral therapy [135]. Thus, there is a large body of evidence rationalising the development of mucosal HIV vaccines [25, 136].

BCG was initially administered orally to children until as recently as 1976 [137] when it was largely replaced by intradermal and percutaneous vaccination regimes. Despite this change, oral vaccination remains an attractive option due to its simplicity, non-invasive nature and potential reduction on costs of immunizations. This is a particularly important consideration in attempts to develop affordable HIV vaccines as the majority of countries where HIV prevalence is high are poor with scarce resources. Several attempts to induce mucosal immunity in experimental animals *via* oral vaccination with BCG have been made [48, 138-141]. The administration of rBCG *via* gastric gavage in non-human primates [71], demonstrated the feasibility of developing an effective oral rBCG vaccine for clinical trials. Formulation of oral BCG with lipids improved protection from TB challenge [139, 142]. Therefore, development of immune responses to oral immunization with BCG and recombinant antigens may be more efficient if the bacilli can be protected from degradation by proteolytic enzymes in the gastrointestinal tract. Formulation of BCG has been attempted. Mice orally vaccinated with BCG trapped in alginate (a naturally occurring polysaccharide) microspheres with a predominant size of 11.5 μm induced strong Th1 BCG immune responses in the spleen [143]. BCG-specific immune responses induced by the entrapped BCG were more potent than those induced by free BCG given orally or BCG given by the subcutaneous route. Formulation of BCG in a lipid matrix which does not affect bacillus viability, before oral administration has successfully protected guinea pigs from a virulent *M. bovis* challenge [144,145]. Protection of the bacillus from proteolytic degradation and movement of lipid encapsulated BCG to mesenteric lymph nodes after oral dosing lead to the development of immune responses to BCG in mice. Such responses were not achieved without formulation [144, 146].

Kawahara and colleagues were able to obtain better cellular and humoral immune responses when immunizing guinea pigs *via* a combination of routes, intra-rectal and subcutaneous or intra-rectal and intra-dermal than when using intra-rectal immunization alone [147]. Langermann *et al*. compared intra-nasal (10^8 cfu x 2), intra-gastric (10^7 cfu) and intra-peritoneal (10^8 cfu) routes of immunization in mice vaccinated with rBCG-expressing the outer surface protein A (OspA) of *B. burgdorferi* fused to the 19kDa leader sequence [147,148]. Intra-nasal and intra-gastric immunization led to IgA responses in the lungs, intestinal lamina propria (LP) and vagina. No mucosal responses were detected following intra-peritoneal immunization. Lagranderie *et al*. found that intra-rectal immunization of mice with rBCG-SIV_{mac251} gave higher intestinal IgA responses than oral or nasal immunization [43]. However, the practicalities of administering a rectal vaccine to humans will probably preclude this route if immunization. Safety issues concerning administering a live, bacterial vaccine intra-nasally is also a concern.

BCG Strain

It has been proposed that a possible explanation for the variable efficacy of BCG lies in the use of different strains of BCG. Various strains have been studied and deletions, insertions and some single nucleotide polymorphisms (SNPs) identified. Lagranderie *et al*. compared the immuno-genicity of five different strains of BCG (Glaxo 1077, Japanese 172, Pasteur 1173P2, Prague and Russian strains) [149]. The Japanese and Prague strains showed reduced ability to multiply and persist in mice, generated weaker immune responses and were less effective in eliminating rBCG expressing β-galactosidase. Wu *et al*. evaluated recall

responses to culture filtrate proteins from BCG Moreau, Denmark and Japan in PBMCs of neonates vaccinated with these strains 1 year post vaccination [150]. High levels of cytokines characteristic of an adaptive immune response were found in children vaccinated with BCG Moreau and Denmark, whereas children vaccinated with BCG Japan showed greater expression of cytokines characteristic of a proinflammatory immune response. However, in another study Horwitz *et al.* examined the protective efficacy of six different strains of BCG (Japan, Danish, Glaxo, Connaught, Pasteur & Tice) in a guinea pig model of TB and found little difference [151]. There are also differing reports on whether the specific strain of BCG can influence expression of foreign genes. Burlein and colleagues found that expression of foreign genes from the same plasmid varied between different BCG strains [152], whereas Al-Zarouni and Dale saw no difference in expression of foreign antigen in three different BCG strains [109].

BCG is contraindicated for HIV positive individuals because it can cause disseminated disease [153]. In a study carried out on SIV-infected macaques, BCG infection led to T-cell activation and marked increases in viral load [154]. In order to render BCG safe for use in immunocompromised people various groups have generated BCG mutants with restricted replication capacity. Tullius *et al*. constructed two mutant strains of BCG: rBCG(*mbtB*)30 which contains an *mbtB* deletion disrupting the synthesis of mycobactin thus preventing normal iron acquisition, and rBCG(*panCD*)30 which is a panothenate auxotroph [155]. Both mutants express the *M. tuberculosis* 30 kDa major secretory protein and grow normally *in vitro* in the presence of supplements but have limited replication *in vivo*. Both strains were safer than BCG in SCID mice and showed limited replication in human macrophages in the absence of supplements. rBCG(*mtbB*)30 provided better protection in guinea pigs, whilst rBCG(*panCD*)30 provided similar protection to BCG. Our group has investigated a pantothenate auxotrophic strain of BCG,(obtained from W.R. Jacobs Jr), as an attenuated vaccine vector strain for HIV. In addition, our laboratory has preliminary data showing that this strain expressing HIV Gag induces a better CD8+ T cell response to Gag than the Pasteur stain of BCG.

Another approach researchers have used in order to develop more immunogenic and effective strains of BCG is to engineer them to escape from the endosome and promote antigen translocation into the cytoplasm. RecBCG, a recombinant UreaseC-deficient BCG strain expressing membrane perforating listeriolysin of *Listeria monocytogenes*, shows significantly improved protection over the parental BCG strain against aerosol challenge with *M. tuberculosis* [7]. It has been suggested that listeriolysin promotes antigen translocation into the cytoplasm as well as apoptosis of infected macrophages, leading to efficient cross-priming and induction of a stronger CD8 T cell response. The Global TB Vaccine Foundation (Aeras) has generated a number of mutant BCG strains based on the expression of perfringolysin O (Pfo) normally secreted by *Clostridium perfringens*, which facilitates escape of the bacteria from the endosome [156]. These strains include a panthothenate auxotrophic strain of BCG that expresses Pfo. It has been demonstrated that BCG expressing Pfo is more immunogenic than wild type BCG as reported by Magalhaes *et al.* using a candidate tuberculosis vaccine tested in rhesus macaques [156]. Two BCG strains, AFRO-1 expressing perfringolysin and BCG (SSI1331), both expressing three mycobacterial antigens, Ag85A, Ag85B and TB10.4, were tested. Following vaccination with these rBCG, the macaques were boosted twice intra-muscularly with a non-replicating adenovirus 35 (rAd35) expressing a fusion protein composed of Ag85A, Ag85B and TB10.4. Animals primed with AFRO-1 had increased Ag85B-specific IFN-γ production in the whole blood assay one week after the first boost with the rAD35 compared to animals primed with BCG (SSI1331). AFRO-1 was established to induce qualitatively and quantitatively different cellular immune responses as compared with BCG(SSI1331) in rhesus macaques [156]. These studies provide strong evidence that the characteristics of the BCG strain can significantly influence the development of the immune response observed.

POTENTIAL LIMITATIONS OF BCG AS A DELIVERY VEHICLE

Genetic Stability

Instability of rBCG episomal vectors has been reported in many rBCGs as reviewed by Dennehy and Williamson [157], and Bastos *et al.* [127]. As rBCG expressing heterologous proteins often have a reduced replication rate, once the heterologous genes have been deleted the resulting defective rBCG out-grow the original rBCG. Even where rBCG is grown with an antibiotic selection to maintain the episomal vectors, the heterologous gene can be deleted or rearranged [158]. This presents a major problem for manufacture of such a vaccine. The quantity of the recombinant protein expressed by a rBCG is a net result of a number of steps; the efficiency of translation, post-translational modification of the proteins, protein folding and the rate of breakdown by proteases. Incorrect folding of proteins could result in increased stress responses and enhanced protease degradation [159]. Most of the recombinant proteins expressed in rBCG at high levels are of bacterial origin such as *E. coli* β-gal (10% of total BCG protein) [39], pneumococcal PspA (15% of total BCG protein) [148] and *B. burgdorferi* OspA (10% of total BCG protein) [11, 160]. The expression of viral antigens in BCG, however, rarely reaches the level of 1% of total BCG protein [32, 35, 98]. Fuerst *et al.* observed that β-gal was expressed to about 15% of total BCG protein, but HIV-1 gp120 was expressed at levels that were 200 times lower, even though the same BCG expression vector was utilized [160]. This is problematic, as low levels of viral antigen expression are likely to result in poor immune responses. Furthermore, some HIV antigens appear to be inherently less stable than others. HIV envelope protein is particularly unstable and can only be expressed in BCG in various truncated forms [43,70-72,74,98,147]. Lim *et al.* reported that rBCG carrying SIV Mac251 *env* encoding amino acids 1-245 was stable but the same vectors containing amino acid 1-521 or 215-521 were unstable [45]. Considerably lower levels of Nef protein were detected from rBCG expressing Nef and Gag although the genes were transcribed off a single mRNA, suggesting that the Nef protein was less stable than Gag [98]. Similarly, Cayabyab and colleagues found that they were able to express high levels of full length SIV Gag utilizing an

episomal plasmid and the ά-antigen promoter whereas a significant proportion of the SIV Pol expressed utilizing the same system, was truncated. Thus, it is possible to improve the stability of a protein by removing regions that are toxic to mycobacteria.

As discussed earlier in the section covering the choice of vector, integrative vectors are usually more stable than episomal vectors. The stability of expression of β-galactosidase was studied in *Mycobacterium smegmatis* using an integrative (pMV361: : lacZ) and a replicative (pMV261: : lacZ) vector [161]. Loss of β-galactosidase occurred at a frequency of 1.7×10^{-5} in the integrative vector and at 2×10^{-3} in the replicative vector. The loss of expression in the integrative vector was due to insertion of an IS element in the *lacZ* gene whereas loss of β-galactosidase activity in the replicative vector was due to deletions within the *lacZ* gene or the *hsp60* promoter. Integrative vectors are better retained than episomal vectors and less likely to be mutated due to their location in the genomic DNA. The lower copy number of integrative vectors leads to lower expression of the recombinant antigen, thus reducing the metabolic load and toxicity imposed on the bacterium. Various groups have improved the stability of episomal vectors by utilizing auxotrophic strains of BCG and including the complementing gene on the vector [91,162]. Using this system of auxotrophic complementation, Nascimento *et al*. were able to remove the antibiotic selection marker and show expression of a pertussis toxin, immunogenicity and protective efficacy [163]. This approach increases the plasmid stability *in vivo* and adds an additional safety feature to the vaccine.

Variable Efficacy of BCG as a TB Vaccine

When BCG is administered at birth it confers consistent and reliable protection against disseminated disease in childhood but variable efficacy against pulmonary tuberculosis (0 to 80%) [164]. The efficacy of BCG is consistently lower in many tropical regions of the world [165]. One of the most popular explanations for the variable protective efficacy of BCG in tropical regions is the masking or possible antagonistic influence of environmental mycobacteria on subsequent BCG vaccination. Similarly, exposure to high levels of tuberculosis has been proposed to mask the effect of BCG. Another explanation that has been proposed is the genetic variation of BCG strains used in different regions. However this theory is not supported by trials carried out in the UK and Malawi where the same BCG strain has shown differing efficacy. There are a number of novel TB vaccines in development that use modified BCG or *M. tuberculosis*. These novel vaccines should be taken into consideration when designing HIV vaccines using BCG as a vector.

Immunosuppression Due to Environmental Mycobacteria

A number of animal studies have provided evidence that sensitization with environmental mycobacteria might have an antagonistic effect on BCG vaccination. De Lisle *et al*. investigated the efficacy of BCG vaccination on guinea pigs presensitised to mycobacteria by oral administration of two different strains of *M. avium* [166]. One strain of *M. avium* decreased the protective effect of BCG vaccination against a virulent *M. bovis* challenge whereas the second strain of *M. avium* had no effect. A similar experiment was carried out in mice. Both strains of *M. avium* were found to be capable of decreasing levels of IFN-γ secretion by splenic lymphocytes although the effect was greater for one strain, WAg 206 [167]. This strain caused a persistent systemic infection whereas the other strain did not persist in mice. Another study showed that presensitisation of mice with environmental mycobacteria can inhibit BCG multiplication and thereby reduce its protective efficacy against TB [168].

However, other studies using mice [169] and guinea pigs [170] have shown no evidence that environmental mycobacteria interfere with BCG protection. Demangel *et al*. investigated the impact of sensitization with *M. avium*, *M scrofulaceum* or *M. vaccae* on the protective efficacy of rBCG expressing RD1 antigens (BCG: : RD1) using a mouse model of TB [171]. Clearance of BCG: : RD1 from the lungs and spleens of mice presensitized with environmental bacteria was minimal compared to that of BCG. Mice sensitized with *M. avium* and vaccinated with BCG: : RD1 showed increased protection against TB compared to BCG. However, both BCG and BCG: : RD1 showed increased protection against TB following sensitization with *M. vaccae* or *M. scrofulaceum*. These results suggest the antagonistic effect of environmental mycobacteria on the protective efficacy of BCG depends on the cross-reactivity of antigens shared with the vaccine. An in silico analysis carried out by Demangel and colleagues showed that the major T cell antigens expressed by BCG are conserved in *M. avium* suggesting that a cross-reactive response would be higher risk than with *M. vaccae* or *M. scrofulaceum* which are phylogenetically more distant.

Anti-Vector Immunity

Pre-existing immunity to vaccine vectors has been shown to have a major impact on the immune response to the viral vaccine vectors adenovirus and MVA, limiting their use in immune populations, or resulting in lower immune responses or the need for higher dosages [172, 173]. Therefore, as for any vaccine vector, BCG immunity arising through vaccination at birth needs to be considered. We have found that when baboons are given several vaccinations with rBCG expressing Gag, there is an increase in the response to Gag after the second vaccination, suggesting that immunity to BCG does not inhibit responses to the recombinant protein (unpublished data). Yu *et al*. found that a second dose of rBCG expressing the HIV-1 envelope protein boosted both HIV-1 envelope and mycobacterial T cell responses in mice [50]. Similarly, strong cellular immune responses to the BCG vector in rhesus monkeys inoculated with rBCG expressing SIV Gag, Pol or Env could be boosted by a second BCG inoculation [74]. HIV specific responses were weakly boosted by a homologous rBCG boost. However the data is conflicting in animal models as some studies show multiple BCG vaccinations can reduce protection. Guinea pigs challenged with M. tb after receiving a single dose of BCG lived for a year, whereas animals receiving 3 doses of BCG 3 weeks apart began to die by week 37 [50]. However, the adverse response seen in the multiply vaccinated guinea pigs could have been due to the frequent timing of the BCG

inoculations and the short interval to challenge. Revaccination of calves with BCG also reduced the level of protection against challenge with *M. bovis* induced by a single BCG vaccination [174], but increased protection in a deer model.

Barreto *et al.* reviewed the literature over the last 50 years and concluded that there is some evidence that a second dose of BCG vaccine does not increase its protective efficacy in humans [175]. However, there does not appear to be any conclusive clinical data indicating that a second dose of BCG increases the incidence of TB. Thus there is no data in humans to indicate whether pre-existing immunity to BCG could reduce the immune response to further doses of rBCG or not.

However, there is data indicating that helminth and Schistosomiasis infections can affect the immune response to BCG and thus immune responses to a rBCG-HIV vaccine might also be affected. Crampin and colleagues suggest that the differences in how infants respond to BCG vaccination in Malawi and the UK could be because the immune system is configured and maintained differently in an African compared to a European setting, leading to implications for both the induction and maintenance of vaccine-induced immunity [176]. Chronic infections, the most common being helminth infections, are very common in tropical regions. Various case-control studies have shown a strong association between intestinal helminth infection and active pulmonary TB [177-179]. In addition, mice vaccinated with BCG in the presence of a chronic helminth infection showed less protection against TB as compared to controls without helminth infection [180]. As BCG is given at birth one could argue that its efficacy should not be affected by helminth infection. However, Malhotra and colleagues have demonstrated that the immunogenicity of BCG vaccination in children born to mothers with Schistosomiasis is poor compared to children born to mothers without Schistosomiasis [181]. This shows that exposure to worm antigens while in the uterus could significantly impair responses to vaccination given after birth.

CONCLUSION

As can be seen from this review, BCG shows great promise as an HIV vaccine vector. Due to its low cost and excellent immunological properties it may be a vaccine of choice to prime the immune system. A number of studies have shown that the protective capacity of a rBCG-vectored HIV vaccine is greatly enhanced when used in a heterologous prime-boost combination, with BCG as the prime. Studies of long term non-progressors suggest that T cells producing multiple cytokines are important for effective protection. This type of T cell response has been shown in monkeys after a prime-boost vaccination with rBCG-rAd5 expressing HIV antigens [74]. In addition a rBCG-SIVgag/rDIs-SIVgag prime/boost combination has been shown to protect against a mucosal challenge with SHIV KS661c in macaques [73].

However, some HIV antigens, such as HIV Env, are poorly expressed in BCG and the rBCG is genetically unstable. Both these factors can result in an rBCG that does not induce optimal immune responses to the HIV antigen. We hope this review has provided some insight on how to overcome problems associated with genetic instability and low immunogenicity in rBCG HIV vaccines. Codon optimisation of the recombinant gene, the choice of vector backbone, the targeting of the recombinant antigen, the use of inducible expression systems are all important factors to consider in when constructing a rBCG vaccine.

ABBREVIATIONS

rBCG = Recombinant *Mycobacterium bovis* BCG

M. tb = *Mycobacterium tuberculosis*

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Received: January 5, 2010 **Revised: January 29, 2010** **Accepted: February 5, 2010**

PMID: 20353397