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The Kinetics of Specific Immune Responses in Rhesus Monkeys Inoculated with Live Recombinant BCG Expressing SIV Gag, Pol, Env, and Nef Proteins

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Development of an effective preventive or therapeutic vaccine against HIV-1 is an important goal in the fight against AIDS. Effective virus clearance and inhibition of spread to target organs depends principally on the cellular immune response. Therefore, a vaccine against HIV-1 should elicit virus-specific cytotoxic lymphocyte (CTL) responses to eliminate the virus during the cell-associated stages of its life cycle. The vaccine should also be capable of inducing immunity at the mucosal surfaces, the primary route of transmission. Recombinant Bacille Calmette–Guérin (BCG) expressing viral proteins offers an excellent candidate vaccine in view of its safety and ability to persist intracellularly, resulting in the induction of long-lasting immunity and stimulation of the cellular immune response. BCG can be administered orally to induce HIV-specific immunity at the mucosal surfaces. The immunogenicity of four recombinant BCG constructs expressing simian immunodeficiency virus (SIV) Gag, Pol, Env, and Nef proteins was tested in rhesus macaques. A single simultaneous inoculation of all four recombinants elicited SIV-specific IgA and IgG antibody, and cellular immune responses, including CTL and helper T cell proliferation. Our results demonstrate that BCG recombinant vectors can induce concomitant humoral and cellular immune responses to the major proteins of SIV.

INTRODUCTION

An ideal vaccine should be safe, stable, inexpensive, and able to elicit immune responses appropriate for the clearance or inhibition of the pathogen in question. Cellular rather than humoral immune responses are more effective in clearing cell-associated virus infection (Rouse *et al.,* 1988). Conversely, many extracellular parasites are most effectively removed by the humoral component of the immune system (Ziegelbauer, 1993). Since HIV-1 and simian immunodeficiency virus (SIV) life cycles have both intracellular and extracellular stages and are primarily transmitted through mucosal surfaces, an effective vaccine should be capable of inducing systemic and mucosal immunity including both cellular and humoral immune responses.

The live attenuated strain of *Mycobacterium bovis,* Bacille Calmette–Guérin (BCG), has a long history of successful vaccine application in both human and nonhuman primates. It has been proven safe in various vaccine trials and, indeed, many countries, with the exception of the United States, exercise routine vaccination with BCG for protection against tuberculosis (Guerin, 1991; Sedaghatian and Kardouni, 1993). BCG has also been successfully used to nonspecifically stimulate cell immunity as a treatment for superficial bladder cancer and skin melanomas (D'Ancona *et al.,* 1991; Lamm, 1995; Mack and Frick, 1995; Melekos *et al.,* 1993; Thanos *et al.,* 1995). Since its initial use as a vaccine against tuberculosis in 1921, more than two billion individuals have been vaccinated with a very low incidence of reported complications (Lotte *et al.,* 1984, 1988; Luelmo, 1982; UNICEF, 1985). Intradermal and oral administration of BCG to newborns as well as to adults have provided long-lasting immunity due to BCG's ability to persist and replicate in macrophages for several decades (Appelberg, 1992; Dannenberg, 1994; Fine *et al.,* 1994; Pithie *et al.,* 1992). Moreover, BCG is well suited for application in developing countries, principally for its inexpensive production cost and heat stability.

Since HIV-1 and SIV infections are primarily cell-associated, it is improbable that a humoral response alone can prevent viral spread. Previous HIV-1 and SIV vaccines geared toward the induction of virus neutralizing antibodies have had limited success (Berman *et al.,* 1988; Hu *et al.,* 1987). In murine influenza and cytomegalovirus systems, cytotoxic T lymphocytes (CTL) have been capable of eliminating or stopping the spread of the virus (Lukacher *et al.,* 1984; Quinnan *et al.,* 1982; Yap *et al.,* 1978). Other studies have reported CTL inhibition of HIV and SIV replication *in vitro* (Kannagi *et al.,* 1988;

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Tsubota *et al.,* 1989; Walker *et al.,* 1987), and have shown that virus-specific CTL can delay disease onset (Koup *et al.,* 1994; Miller *et al.,* 1990; Safrit *et al.,* 1994). Because of its innate adjuvant properties and the potential to stimulate CTL against HIV and SIV infections, BCG provides an ideal vaccine vehicle that should also induce CTL at mucosal sites (Dimov *et al.,* 1992; Gheorghiu, 1994; Munk *et al.,* 1994; Stover *et al.,* 1994).

Advances in recombinant DNA technology allow expression of foreign proteins in BCG (Aldovini and Young, 1991; Barletta *et al.,* 1990; Murray *et al.,* 1992; Ranes *et al.,* 1990; Stover *et al.,* 1991). When BCG was used for the expression of Leishmania surface protein gp63, effective immunity was generated against challenge with infectious Leishmania (Cohn, 1997). In addition, several other groups have engineered HIV and SIV proteins to BCG and detected cellular and/or humoral responses in various animal systems (Aldovini and Young, 1991; Fuerst *et al.,* 1992; Kameoka *et al.,* 1994; Winter *et al.,* 1991; Yasutomi *et al.,* 1993). However, a recombinant BCG expressing a SIV gag single epitope failed to protect macaques against intravenous challenge with cell-free SIV (Yasutomi *et al.,* 1995).

We have studied the immune response to SIV and HIV-1 and have focused in the development and testing of AIDS vaccine candidates (Torres *et al.,* 1993; Anderson *et al.,* 1994; Meyer *et al.,* 1998; Meyer and Torres *et al.,* 1999). In this study, the immunogenicity of recombinant BCG (rBCG) expressing several SIV proteins was tested using the SIV rhesus macaque animal model. For the first time, rBCG constructs containing the four major open reading frames of the SIV genome (*gag, pol, env,* and *nef*) were combined in a single inoculum and given to rhesus macaques intravenously (iv). The results demonstrate the induction of SIV-specific systemic IgA and IgG antibody and cellular immune responses including CTLs and helper T cell proliferation.

RESULTS

Cloning and expression of rBCG-Gag, Pol, Env, and Nef. The entire coding sequences of SIV *gag, pol,* and *nef* were separately subcloned into a BCG–*Escherichia coli* shuttle vector that replicates extrachromosomally. The SIV genes were placed under the regulatory control of the HSP70 promoter that was constitutively active. The *env* gene was subcloned similarly to the other genes except the potentially toxic, hydrophobic signal peptide and transmembrane segment were removed. We therefore expected to see a truncated version of Env in the Western blots. Initial screening for protein expression in *E. coli* was done using Western blot and polyclonal and monoclonal antibodies specific for the respective proteins. The vectors validated for expression of the appropriate proteins were electroporated into BCG and colonies were screened for SIV protein expression. Figure 1 shows the expression of SIVmac Env, Nef, Gag, and Pol detected by macaque polyclonal antibodies. The molecular weight of nef protein correlates with the respective proteins in the SIVmac239 virus lysate and that of env, with the size predicted from the gene truncated at the 5′ and 3′ ends. Bands were also obtained for recombinant BCG expressing HIV Gag and Pol (lanes 5 and 6). No reactivity with proteins from mock transfected BCG was detected (lane 2). Lane 7 shows a band pattern demonstrating specific binding of SIVmac proteins by the polyclonal antibodies.

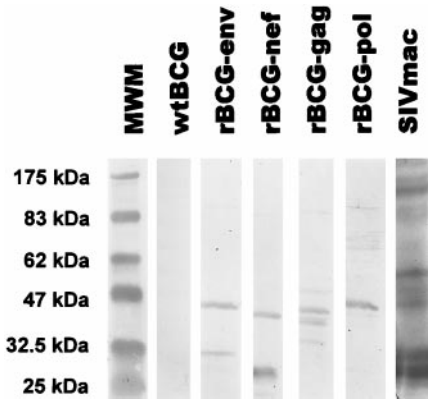

FIG. 1. SIV protein expression by recombinant BCG. Western blot analysis of rBCG expressing SIV Env, Nef, Gag, and Pol proteins. The samples were prepared as described under Materials and Methods. Specific SIV proteins were detected by 1:800 dilution of polyclonal sera from an SIV-infected rhesus macaque. Antigens: Lane 1, molecular weight markers; Lane 2, wild-type BCG; Lane 3, rBCG-env; Lane 4, rBCG-nef; Lane 5, rBCG-gag; Lane 6, rBCG-pol; and Lane 7, SIVmac.

SIV-specific lysis of rVV-labeled B-LCL targets. Peripheral blood mononuclear cells (PBMCs) from six inoculated rhesus macaques were tested for their ability to lyse autologous B-LCL targets expressing SIV proteins. Prior to the assay, PBMCs were nonspecifically stimulated with Con A for 3 days and subsequently expanded in IL-2 medium for an additional 3 days. As seen in Fig. 2, specific CTL activity was detected against one or all the SIVmac proteins in the immunized rhesus macaques. No CTL was detected in the nonimmunized controls (data not shown). One macaque was sacrificed a month after rBCG inoculation and the remaining five animals were kept until the end of the study. By 30–34 days following rBCG inoculation, specific lytic activity to SIVmac Pol and Env proteins but not SIVmac Gag was detected in Monkey 1 (Fig. 2). The other five macaques (Monkeys 2–6) also had CTLs specific for more than one SIVmac protein present at several time points. CTL responses to Gag, Pol, and Env proteins were demonstrated for Monkey 2 at 41, 158, and 180 days after rBCG inoculation. For Monkey 3, CTLs specific for Pol and Env proteins were detected at 25 days and to all three proteins tested at day 137. CTLs to envelope were present in Monkey 4 at both 25 and 159 days postinoculation. CTL

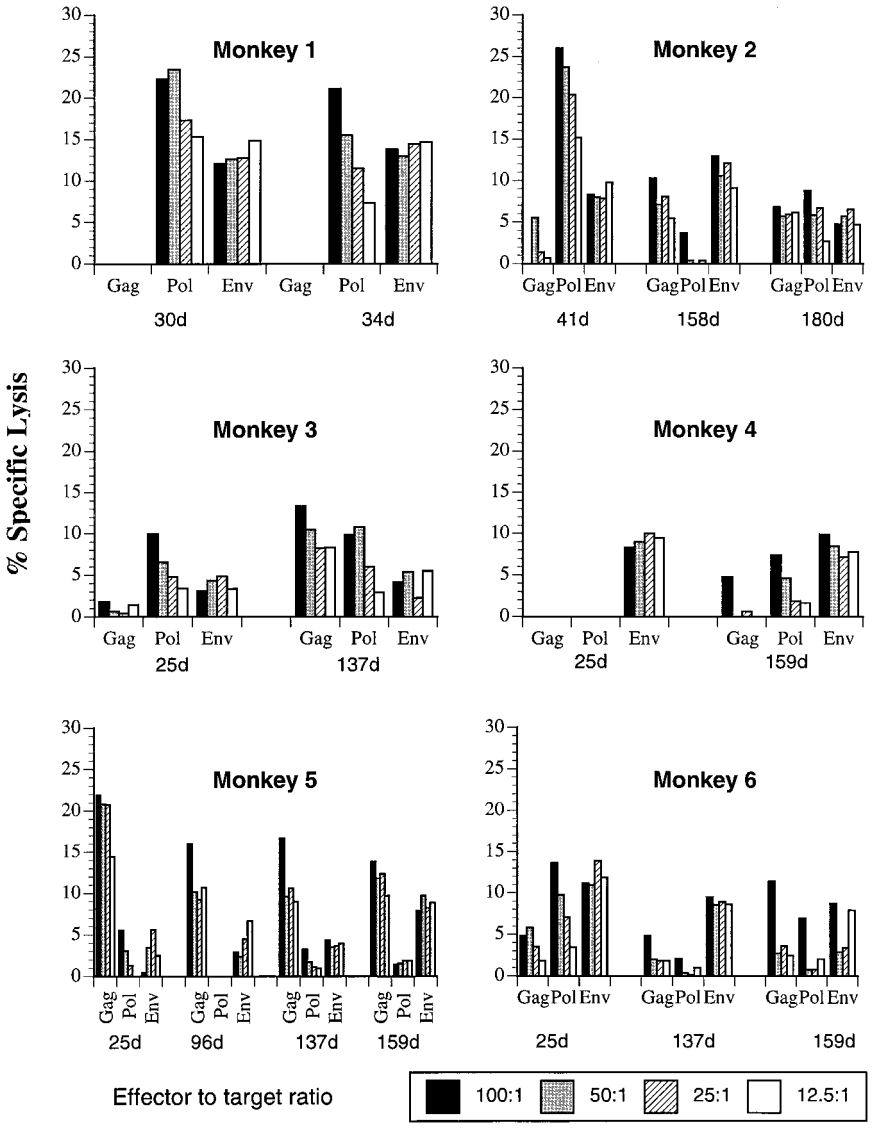

FIG. 2. CTL response induced by recombinant BCG. PBMCs from rBCG-inoculated rhesus macaques specifically lyse B-LCL targets labeled with rVV expressing SIVmac251 Gag, Pol, or Env proteins. PBMCs were assayed at several dates postinoculation as indicated on the *x* axis. B-LCL targets infected with wild-type vaccinia were used as negative controls. The percentage specific release shown is after subtraction of wild-type rVV values. Spontaneous release was less than 20%.

activity specific for Gag, Pol, and Env proteins were detectable as early as 25 days and remained high at 159 days in Monkeys 5 and 6 (Fig. 2). Overall, consistent CTL responses to SIVmac Gag, Pol, and Env in PBMCs of five of the six vaccinated monkeys were demonstrated in the study.

SIV-specific IgA and IgG response. The PBMCs of the rBCG-inoculated rhesus macaques were tested at different times postinoculation and varying levels of SIV-specific IgA and IgG secretion were detected in all the immunized monkeys (Fig. 3). One macaque (Monkey 7) was an infected control. IgA and IgG levels specific for both recombinant envelope glycoprotein (gp130) and whole SIV antigen were secreted by PBMCs harvested from all the animals tested (Monkeys 2–6). A panel of uninfected macaques was used to establish the baseline control values (<0.2 OD units at 405 nm). In addition, these assays were also performed using PBMCs from the study animals before immunization to confirm baseline values. Data presented in Fig. 3 show that these responses increase over time after immunization for the duration of the testing period. With the exception of IgG levels to whole SIV, both IgA and IgG levels to gp130 in the immunized animals were higher than those found in the infected control (Monkey 7).

Additional immunological assessment. Further analysis of the immune response induced by the recombinant BCG expressing SIV gag, pol, env, and nef revealed detectable antibody levels in some animals. Four out of the six animals were positive for antibodies to purified

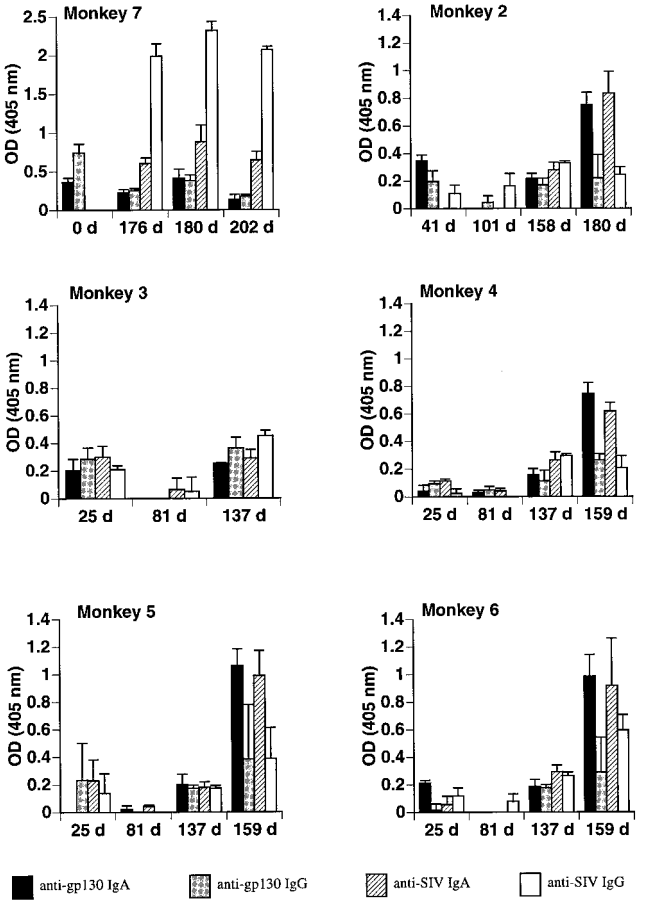

FIG. 3. Humoral response following recombinant BCG inoculation. IgA/IgG secreted by PBMCs from rBCG-inoculated rhesus macaques. PBMCs were assayed by cell ELISA at several dates postinoculation as indicated on the *x* axis. An SIV-infected rhesus macaque was used as a positive control (Monkey 7). Error bars indicate variation among individual wells for each macaque. All assays were done at least in triplicate. The upper baseline value of 0.2 OD at 405 nm established with a panel of uninfected macaques was subtracted from each data point.

protein derivative by ELISA and all the animals were positive for antibodies specific for BCG by Western blot. ELISA and Western blot analysis showed that three macaques produced detectable antibodies to our SIVmac nef expressed in *E. coli.* Furthermore, detectable titers of SIV neutralizing antibodies were observed in four of the six macaques.

DISCUSSION

With advances in rDNA technology, BCG can be engineered to express foreign proteins (Aldovini and Young, 1991; Stover *et al.,* 1991). Other desirable characteristics, such as its long safety record in humans, stability, low cost, and particularly its innate ability to stimulate CTL production, make rBCG expressing HIV-1 proteins an ideal vaccine candidate against AIDS. Information regarding the dose, route, immunogenicity, safety, and efficacy of BCG immunization against tuberculosis in rhesus macaques, combined with the availability of the SIV rhesus macaque animal model of simian AIDS, prompted us to evaluate the potential of rBCG as a vaccine against human AIDS (Barclay *et al.,* 1973; Chaparas *et al.,* 1975; Good, 1968). Preexisting immunization with BCG has not been shown to interfere with the use of rBCG as a vaccine vector nor BCG used as an immune adjuvant in the treatment of certain carcinomas.

SIV infection of rhesus macaques is an excellent model for AIDS in humans. SIV and HIV-1 have similar genetic organization and cell tropism for the CD4 receptor and induce a similar AIDS-like disease in macaques and humans, respectively. The main difference is a shortened disease course for SIV, (6 months–2 years in macaques) in comparison to HIV-1 (6–10 years in humans) (Kangnoff, 1978; Kuller *et al.,* 1990; Letvin *et al.,* 1985; Letvin and King, 1990).

We have tested the immunogenicity of rBCG expressing SIV Gag, Pol, Env, and Nef proteins in rhesus macaques by administering all four recombinants in a single inoculation. The dose was chosen from an early study evaluating the safety and efficacy of inoculated native BCG as a vaccine against simian tuberculosis (Good, 1968). More recent studies with rBCG expressing either SIV Gag or Nef proteins inoculated intradermally were successful in eliciting SIV-specific CTL (Yasutomi *et al.,* 1993). The objective of the present study was to determine whether simultaneous inoculation of all four rBCGs would elicit immune responses to all four SIV proteins (Gag, Pol, Env, and Nef), and, if so, to identify the pattern and duration of the responses. Recombinant BCG expressing Nef was specifically included since Nef is one of the earliest proteins expressed during natural HIV-1/SIV infection and is important in maintaining a high virus load *in vivo* (Ameisen *et al.,* 1989; Koenig *et al.,* 1990). Thus, the induction of CTL immune responses against Nef may be important in controlling virus spread. Unfortunately, because the recombinant vaccinia expressing SIV Nef was unavailable to us, we were unable to determine whether CTLs against Nef were present. It is highly unlikely, however, that CTL responses would be generated against only Gag, Pol, Env, and not Nef, particularly in light of previous studies that show Nef is immunogenic (Bourgault *et al.,* 1992; Culmann *et al.,* 1991; Koenig *et al.,* 1990; Lamhamedi-Cherradi *et al.,* 1992; Venet *et al.,* 1992; Yasutomi *et al.,* 1993). In addition, we performed ELISA and Western blot assays that demonstrated a humoral response against *E. coli*-expressed Nef in three of the monkeys (data not shown). Purified recombinant Nef expressed in *E. coli* was prepared by our lab for use in these experiments. Therefore, rBCG was able to induce a Nef-specific humoral immune response in at least 50% of the animals.

Outbred populations with extensive MHC polymorphism will develop CTLs against one or more but not

necessarily all HIV-1 or SIV proteins, and the recognition pattern can change over time (Phillips *et al.,* 1991; Walker *et al.,* 1987). Therefore, vaccine strategies against AIDS should generate immune responses against multiple viral components as well as account for escape mutants that frequently arise in the course of HIV-1 and SIV infection (Phillips *et al.,* 1991). Our data highlight differences in the kinetics of CTL responses directed against major proteins of SIV when expressed by a live vector *in vivo.*

Interestingly, we found that early after inoculation of the macaques with the four recombinant BCGs (approximately 4 weeks), the dominant CTL responses were directed against Pol and Env (in 4/6 monkeys) as opposed to Gag (in 1/6 monkeys). This is somewhat surprising given that the Gag-specific CTL response has been characterized as the dominant CTL epitope after SIV-infection of rhesus macaques (Egan *et al.,* 1999). A previous study examined CTL responses over time against SIVmac Gag, Env, and Nef after infection of rhesus macaques with SIVmac (Yasutomi *et al.,* 1993). The earliest CTL response was directed against Env (within 1 week) and later against Gag and Nef (at 4 weeks postinfection), confirming our results regarding an early Env-specific CTL response. One possible explanation for a slower Gag-specific response in our study is that the Gag-expressing rBCG replicated at a slower rate *in vivo* relative to the other rBCGs, thus delaying induction of a Gag-specific CTL response. We find this unlikely, however, as one of our monkeys mounted a very potent Gag-specific CTL response and minimal Pol- and Env-specific responses early after inoculation (Monkey 5). Thus, we believe that inoculation of macaques with live rBCGs expressing multiple SIV antigens, or infection with live SIVmac, leads to early anti-SIV responses directed against Pol and Env and later against Gag.

The importance of the kinetics of CTL responses directed against the various proteins of SIV and HIV in relation to protection from infection of disease is not yet clear. One study found that, among recently seroconverted HIV-infected individuals, the earliest CTL responses are directed against Gag and Env, with little reactivity against HIV-1 Pol (Legrand *et al.,* 1997). In contrast, another longitudinal and cross-sectional study examined CTL responses in relation to the vertical transmission of HIV-1 (Jin *et al.,* 1998). Limiting dilution analysis was used to quantitate CTL frequencies against HIV-1 Gag, Pol, Env, and Nef in 15 women who transmitted disease and in 16 women who did not transfer disease. CTL frequencies directed against Pol and Nef were higher during the pregnancies of nontransmitters compared to those women transmitting virus. Thus, in animal models of HIV infection and among HIV-infected individuals, the kinetics of CTL responses against the major viral proteins differ from one another. Moreover, the viral targets of early CTL responses may impact the clinical course of HIV-1 infection.

Since HIV-1 is generally transmitted sexually via mucosal surfaces, a vaccine should ideally generate mucosal immunity. A major component of mucosal immune responses involves IgA-secreting plasma cells at mucosal sites. Interestingly, detection of a systemic antigen-specific IgA response may be indicative of mucosal immunity (Forrest, 1992). All the inoculated rhesus macaques in this study demonstrated systemic SIV-specific IgA responses which varied over time, generally peaking between 160 and 190 days after inoculation. In fact, virus-specific IgA responses dominated IgG responses at late (160–190 days) time points in inoculated macaques. In marked contrast, virus-specific IgG responses were dominant in an SIV-infected macaque (Monkey 7). It is possible that the extended duration of the IgA antibody response demonstrated here is due to the persistence and replication of the rBCG at both mucosal and nonmucosal sites, thus providing continuous antigenic stimulation. The intravenous route was chosen as an aggressive approach to testing the immunogenicity of the rBCG constructs and is not meant to be suggested as a vaccination route. Studies comparing the immunogenicity of the four rBCGs administered orally, intragastrically, and intradermally are in progress.

HIV-1 and SIV exist as cell-associated and cell-free viruses; therefore, an effective vaccine must generate both cellular and humoral immunity. Modifications in expression of foreign antigens, such as exportation to or away from cell surfaces, may lead to an increase in virus-specific antibody expression. Indeed, significant increases in humoral responses can occur when foreign proteins are directed to the membrane surface or secreted into the extracellular medium, together with cytokines (O'Donnell *et al.,* 1994; Stover *et al.,* 1993). We have also developed a combination approach with SIV-specific peptides and rBCG to elicit both cellular and humoral immunity to SIV/HIV-1 (Appelberg, 1992). Oral administration of rBCG may elicit a stronger local IgA response due to lymphocyte homing mechanisms and the common mucosal pathway (McDermott and Bienenstock, 1979). Recent studies have demonstrated the induction of neutralizing antibodies and T cell proliferation in small animals by rBCG expressing SIV proteins (Lim *et al.,* 1997).

HIV-2 has a similar genomic organization and antigenic profile as that of SIVmac. Therefore, it is logical to assume that an effective vaccine against SIV in rhesus macaques will also protect against HIV-2 in humans. We intend to develop this vaccine for use against HIV-2 infection and disease progression in West Africa. In addition, rBCG as a vaccine vector has recently acquired renewed importance due to the worldwide reemergence of human tuberculosis (Cohn, 1997).

With the SIV/rhesus macaque animal model of simian

AIDS, we have shown that a single inoculation of four individual rBCG vectors expressing SIV Gag, Pol, Env, and Nef proteins can induce SIV-specific CTL directed to more than one protein. Within 1 month of inoculation, CTL responses were directed against Pol and Env, and later against Gag. Furthermore, SIV-specific IgA antibodies were also elicited systemically. The long record of safety of BCG use in humans, the innate adjuvant properties, and the role of CTL in controlling HIV-1 replication and spread suggest that rBCG may yet prove an excellent vaccine vector against AIDS.

MATERIALS AND METHODS

Animals. Healthy adult rhesus macaques (*Macaca mulatta*) were selected and housed at the California Regional Primate Research Center at the University of California, Davis, in accordance with the guidelines established by the American Association for the Accreditation of Laboratory Animal Care (Assurance No. A3433-01). All animals were screened for retroviruses including STLV, SRV, and SIV. Collection of blood samples for PBMCs, serum, and plasma was obtained by venipuncture after sedation with ketamine hydrochloride (10–40 mg/kg).

Construction of rBCG vectors. The construction of the expression vectors pY6013 and pYUB12 has been previously described (Aldovini and Young, 1991; Snapper *et al.,* 1988). Briefly, pY6013 contains the mycobacterial hsp70 promoter and coding sequence; pYUB12 contains the origins of replication for both *E. coli* (P15A) and mycobacterium, as well as the Tn*903*-derived *aph* gene to confer kanamycin resistance. Construction of the rBCG involved a two-step process wherein the gene of interest was first inserted into pY6013 so as to place it in frame and under the regulatory control of the hsp70 promoter. The specific DNA insert was obtained by polymerase chain reaction (PCR). The PCR was performed in a Perkin–Elmer Cetus DNA thermocycler (Perkin–Elmer Cetus, Norwalk, CT). All DNA fragments were generated using SIVmac239-specific primers which were designed to introduce a *Nco*I site overlapping the translation initiation codon at the 5′ end, and a *Xba*I site immediately following the translation stop codon. The reaction mixture contained a total volume of 100 μl, consisting of 100 ng template, 40 pmol of each primer, 2 U *Taq*I polymerase (Perkin–Elmer Cetus), 0.2 mM of each dNTP (Pharmacia, Piscataway, NJ), 10× reaction buffer (500 mM KCl, 100 mM Tris–HCl at pH 8.3, 25 mM $MgCl_2$, 0.1% gelatin), and 50 μl mineral oil. The DNA was amplified for 30 cycles with temperatures for denaturing at 94°C, annealing at 56°C, and extension at 72°C. From pY6013, a 1.8-kb DNA fragment was removed by a *Nco*I/*Xba*I digestion and the PCR-generated DNA *gag, pol, env,* or *nef* fragment was inserted (the translation initiation codon for the hsp70 coding sequence also overlaps a *Nco*I site). The gene of interest and the hsp70 promoter were then excised with an *Eco*RI/*Xba*I digest (the *Eco*RI site was blunt-ended with T4 DNA polymerase) and ligated into the *Eco*RV/*Xba*I sites of the pYUB12 *E. coli*–mycobacterial shuttle vector.

DNA fragments were generated using SIVmac251 or SIVmac239 primers designed to introduce a *Nco*I site at the junction of promoter and open reading frame, and a *Kpn*I or *Xba*I site immediately following the translation stop codon. The following oligonucleotides were used in the synthesis of the different genes: *gag* (5′ to 3′): GGCGCGAGAAACGCCGTCTTGTCA (SIVmac251), AGGGTACCCTACTGGTCTCCTCCAAAGAGAGA (SIVmac251 plus *Kpn*I site); *pol* (5′ to 3′): AACCCGGGAGCCCCAAGAAGACAGAGATG (SIVmac251 plus *Sma*I site), ATCCCGGGCTATGCCACCTCTCTAGCCTCTCC (SIVmac251 plus *Sma*I site); *env* (5′ to 3′): CAGTCTCCATGGGTGTACCAGCTTGG (SIVmac239/251 plus *Nco*I), CCACCTCTAGATTACCTCTTCACATCTGTGG (SIVmac239/251 plus *Xba*I); *nef* (5′ to 3′): CTACAATCCCGGGGGAGCTATTTCCATGAG (SIVmac239/251 plus *Sma*I site), GTCCCTTCTAGATCAGCGAGTTTCC (SIVmac239 plus *Xba*I site).

Gag and Pol primers were synthesized according to the sequence reported previously that relates to SIVmac251 (Accession No. M19499) with some differences (Franchini *et al.,* 1987). The PCR reaction was carried out with *Taq*I polymerase according to the manufacturer's protocol (Perkin–Elmer Cetus). *Gag, pol, env,* and *nef* gene fragments were cloned in plasmid pY6013 containing the HSP70 promoter (Aldovini and Young, 1991). For the *gag* gene, pY6013 was cut with *Nco*I, filled in with Klenow, and digested with *Kpn*I. The plasmid vector without HSP70 coding sequence was selected and ligated with a purified *gag* PCR product that was filled in with Klenow and then cut with *Kpn*I. A recombinant plasmid containing the *gag* gene that recreates the *Nco*I site at the junction of the HSP70 promoter and the *gag* open reading frame was selected. For the *pol* gene, pY6013 was cut with *Nco*I and *Kpn*I and filled in with Klenow. Plasmid vector without HSP70 coding sequence was selected and ligated with a *pol* PCR product digested with *Sma*I. A recombinant plasmid containing the *pol* gene in the correct orientation was selected. DNA fragments covering the *env* and *nef* sequences replaced the HSP70 coding region in pY6013 as *Nco*I–*Xba*I and *Sma*I–*Xba*I fragments, respectively.

The four recombinant plasmids containing SIV genes downstream of the HSP70 promoter were digested with *Eco*RI, filled in with Klenow, and partially (*gag* and *pol*) or completely (*env* and *nef*) digested with *Xba*I. Fragments containing the *gag, pol, env,* and *nef* genes driven by the HSP70 promoter were isolated from the digested plasmids and inserted in the backbone of pYUB12, purified after digestion with *Eco*RV and *Xba*I.

The entire coding sequences of SIVmac239 *gag, pol,* and *nef* were inserted into pYUB12. However, it was necessary to truncate the SIV *env* coding sequence to

remove certain hydrophobic regions since foreign genes overexpressing hydrophobic regions can be toxic to prokaryotic cells. We therefore designed primers based on exclusion of the amino-terminal signal peptide and the hydrophobic transmembrane segment at the carboxyl terminal. The resulting PCR-derived DNA fragment was then inserted into pYUB12.

The DNA constructs were transformed into *E. coli* DH5α and colonies were screened by restriction enzyme analysis. Positive colonies were expanded and the recombinant DNA constructs were purified by cesium chloride banding. Monoclonal antibodies specific for Gag, Pol, Nef, and Env were used to confirm the expression of these antigens in *E. coli.* The rDNA constructs were subsequently electroporated into wild-type BCG and screened for appropriate protein expression by Western blot.

Growth of rBCG. The Pasteur strain of BCG (TMC 1011, American Type Culture Collection (ATCC) No. 35734) was obtained from the ATCC and grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented to a final concentration of 10% albumin–dextrose complement (Difco), 0.05% Tween 80 (Sigma, St. Louis, MO), and 0.2% glycerol (Fisher, Springfield, NJ). The supplemented medium was referred to as complete MB7H9. Kanamycin (Sigma) was used as a selectable marker for the rBCG (20 μg/ml). Bacterial cultures were grown at 37°C until they reached mid-log phase, and were then harvested for electroporation. Briefly, BCG was washed and resuspended to one-tenth their original volume and 0.4 ml was aliquoted into 0.4-cm electroporation cuvettes (Bio-Rad, Richmond, CA). Recombinant BCG DNA constructs were added to the cells (1 μg DNA) and the cuvettes were placed in a Bio-Rad Gene Pulser with an attached pulse controller (Bio-Rad). The BCG constructs were electroporated at 25 μF and 2.5 kV. After approximately 4 weeks, individual colonies were selected and expanded in 25 ml of complete MB7H9 plus kanamycin and grown at 37°C until mid-log phase. rBCG cell lysates were obtained by sonication with a Microson microtip sonicator (Misonix, Farmingdale, NJ) using two 10-s pulses. The rBCG lysates (200 μl) were added to 50 μl of 5× Laemmli's buffer (Laemmli, 1970) and boiled at 100°C for 5 min. The cellular debris was pelleted by centrifugation for 3 min at 10,000 rpm (Savant Instruments, Farmingdale, NY) and 20 μl of each lysate was assayed by Western blot analysis for appropriate protein expression.

Western blot analysis. Lysates from each of the rBCG colonies were screened by Western blotting for SIV protein expression. One milliliter of rBCG at mid-log phase was centrifuged in a microcentrifuge for 1 min at 10,000 rpm and resuspended in 0.5 ml of sterile PBS. Samples were sonicated with a Misonix microtip sonicator using two 10-s pulses, then 2× Laemmli's buffer (Laemmli, 1970) was added and samples were denatured by boiling at 100°C for 5 min. Proteins were resolved in a discontinuous acrylamide gel system consisting of a 10% resolving gel and 5% stacking gel. Proteins were transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) and blocked with Blotto (5% nonfat milk in PBS in 0.1% Tween 20) for 1 h at room temperature. After the membrane was washed three times with PBS containing 0.1% Tween 20, polyclonal sera from an SIVmac251-infected rhesus monkey was added (1:800) and incubated at room temperature for 1 h with gentle agitation. The membrane was washed again and a secondary goat-anti-human antibody conjugated to alkaline phosphatase (Fisher Biotech) was added (1:800). The membrane was again washed and 15 ml of *p*-nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate substrate was added.

Immunization. To prepare for inoculations, 500 ml of each rBCG was grown in Fernbach flasks and harvested at mid-log phase. The cells were tested for lack of bacterial contamination and for viability by staining with both Gram and Kinyoun's cold acid-fast stains (Fisher). To obtain approximately 1×10^8 CFU of each rBCG, a specific volume of each supernatant was harvested based on previous growth curves correlating OD and CFU/ml. The actual concentrations were obtained by titration and compared with the estimated values. Briefly, the rBCGs were titrated by plating dilutions and counting the colonies on Middlebrook 7H10 agar supplemented with 0.5% glycerol, 0.05% Tween 80, and 20 μg/ml kanamycin. The individual rBCGs were subsequently combined and another aliquot was set aside for titration. The combined rBCGs were concentrated in a 5-ml volume in sterile saline for inoculation into rhesus macaques.

Rhesus macaques were inoculated iv with a high dose of rBCG ($5–7 \times 10^8$ CFU/rhesus) concentrated in a 5-ml volume. According to the guidelines of the study, one macaque was sacrificed after 1 month and the tissues were stored for further analysis.

Cytotoxic T lymphocyte assays. Effector cells: Blood was obtained by venipuncture and lymphocytes were collected by Ficoll–Hypaque density centrifugation (Organon Teknika, Durham, NC). The PBMCs were placed in culture at a concentration of 1×10^6 cells/ml and stimulated for 3 days in complete medium [RPMI 1640 supplemented with 10% fetal calf serum (Gemini Bioproducts Inc., Calabasas, CA)], 2 mM L-glutamine (Sigma), and 50 μg/ml gentamicin sulfate (Irvine Scientific, Santa Ana, CA) containing 5 μg/ml Con A (Sigma). After the 3-day incubation, the PBMCs were washed once with complete medium, and stimulated an additional 3 days in complete medium containing 40 U/ml of recombinant human IL-2 (Chiron Corp., Emeryville, CA). At the end of the 6-day stimulation period, the effectors were centrifuged, washed once with complete medium, and resuspended to 1×10^7 cells/ml. Twofold serial dilutions were performed and 0.1 ml of 1×10^7 cells/ml through $1.25 \times$

10^6 cells/ml dilutions were added to each of four wells in triplicate.

Target cells: Autologous B lymphocyte cell lines (B-LCL) were obtained by transformation of macaque PBMC with *Herpes papio* and subsequently stored in liquid nitrogen (Miller *et al.,* 1990). To generate SIV-specific targets, autologous B-LCL were infected with recombinant vaccinia (rVV) that expressed either SIVmac251 Gag, Pol, or Env as previously described by Letvin (Miller *et al.,* 1990; Yasutomi *et al.,* 1996). The recombinant vaccinia virus expressing SIV Nef was not available. Cells were infected at a multiplicity of infection of 5 PFU/cell and incubated at 37°C for 16 h. They were then washed twice and cell concentration and viability were determined by trypan blue exclusion (Sigma). The target cells were labeled by incubation with 0.2 mCi of $Na_2^{51}CrO_4$ (Amersham) for 1 h at 37°C. The targets were washed three times with complete medium. To obtain effector:target cell ratios of 100:1, 50:1, 25:1, and 12.5:1, 0.1 ml of a 1×10^5 cells/ml target cell concentration was added to effector cells in the appropriate wells. Effector and target cells were incubated for 5 h at 37°C in a 5% CO_2 atmosphere. The cells were then pelleted by centrifugation for 10 min at 200 *g*. Supernatant (100 μl) was collected and counted in a 1260 multigamma counter (LKB Wallac, Finland). The total ^{51}Cr content was obtained by addition of 0.1 ml 5% Nonidet P-40 (Sigma) to wells containing target cells only. Spontaneous release values obtained from wells containing labeled targets and medium only were less than 20%. Specific release was calculated as [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. The calculated specific release values represent the lysis of target cells infected with rVV expressing SIVmac Gag, Pol, or Env minus the specific lysis of the target cells infected with the wild-type vaccinia strain.

IgA/IgG assay. As a marker for potential mucosal and systemic immune responses, SIV-specific IgA and IgG secreted from PBMC were assayed using a modified protocol (Forrest, 1988, 1992). It was previously shown that antigen-specific IgA secreted by human PBMCs 7 days after mucosal immunization was more indicative of mucosal immune responses than IgA from serum, saliva, or intestinal wash (Forrest, 1992). Therefore, to determine if a single inoculation of rBCG expressing SIV Gag, Pol, Env, and Nef would be able to elicit SIV-specific IgA responses, a modification of this IgA ELISA was performed as described below.

Fresh rhesus PBMCs were stimulated on ELISA plates containing either bound inactivated SIV or recombinant gp130 as antigens. Briefly, wells of a 96-well plate were coated with either 200 ng/well of whole-inactivated SIVmac239 or 800 ng/well of SIVmac251 rgp130. The plate was then blocked with Blotto (10% nonfat milk in PBS containing 0.5% bovine serum albumin and 0.05% Tween 20). PBMCs were added at a concentration of 5×10^5 cells/well in 0.1 ml and incubated for 16 h at 37°C in a 5% CO_2 incubator. The plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween 20 and 0.02% sodium azide). To detect secreted IgA or IgG antibodies, rabbit anti-monkey IgA or IgG (Nordic Immunology, Tilburg, The Netherlands) was added at a dilution of 1:1000 and incubated for 4 h at 37°C. The plates were again washed and the tertiary antibody, goat anti-rabbit conjugated to alkaline phosphatase (Fisher Biotech), was added at a dilution of 1:1000 and incubated for 1 h at 37°C. After the plates were washed, 0.1 ml of a 1 mg/ml solution of *p*-nitrophenyl phosphate in 10% diethanolamine buffer (Sigma) was added and the absorbance was read at 405 nm on an EIA autoreader model EL310. Wells were blanked with values from antigen-coated wells incubated with only the alkaline phosphatase conjugated antibody. We also determined that comparison of values obtained from experimental cells incubated with antigen versus experimental cells incubated without antigen was an accurate indicator of antigen-specific antibody secretion. This is significant particularly because the assay requires the use of fresh PBMCs, which makes comparison to preimmunization values difficult due to possible interassay variation. Therefore, the assay was also performed as previously described with the addition of Blotto-coated wells (no antigen) incubated with the PBMCs. In addition, baseline control values were established with a panel of uninfected macaques and were less than 0.2 OD units at 405 nm.

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