Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10693–10697, November 1995
Immunology

Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette–Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals

(principal neutralizing determinant/field isolate of human immunodeficiency virus)

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Communicated by Tadamitsu Kishimoto, Osaka University Medical School, Osaka, Japan, June 26, 1995

ABSTRACT **A recombinant *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vector-based vaccine that secretes the V3 principal neutralizing epitope of human immunodeficiency virus (HIV) could induce immune response to the epitope and prevent the viral infection. By using the Japanese consensus sequence of HIV-1, we successfully constructed chimeric protein secretion vectors by selecting an appropriate insertion site of a carrier protein and established the principal neutralizing determinant (PND)-peptide secretion system in BCG. The recombinant BCG (rBCG)-inoculated guinea pigs were initially screened by delayed-type hypersensitivity (DTH) skin reactions to the PND peptide, followed by passive transfer of the DTH by the systemic route. Further, immunization of mice with the rBCG resulted in induction of cytotoxic T lymphocytes. The guinea pig immune antisera showed elevated titers to the PND peptide and neutralized HIV_{MN}, and administration of serum IgG from the vaccinated guinea pigs was effective in completely blocking the HIV infection in thymus/liver transplanted severe combined immunodeficiency (SCID)/hu or SCID/PBL mice. In addition, the immune serum IgG was shown to neutralize primary field isolates of HIV that match the neutralizing sequence motif by a peripheral blood mononuclear cell-based virus neutralization assay. The data support the idea that the antigen-secreting rBCG system can be used as a tool for development of HIV vaccines.**

Candidate preparations of vaccines against human immunodeficiency virus (HIV) infection have been clinically tested in humans to assess their safety and capacity to stimulate the immune system. These candidate vaccines were based on subunits of viral antigens that were presented with adjuvant, focusing on eliciting production of neutralizing antibodies (1). On the other hand, recent observations in humans demonstrated that cellular immunity is important for some HIV-infected people to remain healthy (2). Although it is not clear what correlates with the viral protection, it is probable that both humoral and cellular immunity contribute to protection from HIV infection or development of AIDS (3). Live vectors have been typically used to obtain such responses. Immunization with recombinant *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) (rBCG) expressing HIV antigens has been reported to induce immune responses against various components of viral antigens (4). However, no such rBCG has been shown to induce efficient protective immunity against HIV infection.

In this study, we established an effective candidate antiviral vaccine by using an antigen-secreting rBCG system that could induce a strong delayed-type hypersensitivity (DTH) reaction, cytotoxic T lymphocytes (CTLs), and neutralizing antibodies to the virus antigen at levels that protected mice against HIV in severe combined immunodeficiency (SCID) mouse models of the virus infection.

MATERIALS AND METHODS

Construction of Expression Vectors and Expression of the Principal Neutralizing Determinant (PND)–α-Antigen Chimeric Protein. A synthetic DNA fragment encoding the 19-aa V3 sequence of Japanese consensus HIV (NTRKSIHIGPGRAFYATGS) was inserted into plasmid pKAH20 (5) that contains the gene for α-antigen of *Mycobacterium kansasii*. The positions of the oligonucleotides are numbered relative to the HXB2 isolate in the Entrez data base, National Center for Biotechnology Information, National Library of Medicine (National Institutes of Health, Bethesda, MD). The sequence comprised nt 7127–7189 plus *Xho* I recognition sites (5′-TCGAGTAACACGAGGAAGAGCATCCACATCGGGCCCGGGAGGGCATTTACGCCACCGGG-3′ for the upper strand with optimal codons used in mycobacteria) and was inserted into an *Escherichia coli*/mycobacteria shuttle vector pIS18 (5) to obtain pIJM-V3-19X (Fig. 1*A*).

DNA fragments encoding the PND of the HIV-IIIB strain (RIQRGPGRAFTIGK) were also synthesized and inserted into the existing *Xho* I/*Pst* I sites in the coding region of the α-antigen in the α-antigen expression vector pIJK-1 (5); the resulting plasmids were designated pIJM-V3-15X and pIJM-V3-15P, respectively. BCG substrain Tokyo and *Mycobacterium smegmatis* strain ATCC607 were transfected with the

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Abbreviations: HIV, human immunodeficiency virus; BCG, *Mycobacterium bovis* bacillus Calmette–Guérin; rBCG, recombinant BCG; PND, principal neutralizing determinant; DTH, delayed-type hypersensitivity; CTL, cytotoxic T lymphocyte; SCID, severe combined immunodeficiency; PBMC, peripheral blood mononuclear cell; KLH, keyhole-limpet hemocyanin; PHA, phytohemagglutinin; mAb, monoclonal antibody; PPD, purified protein derivative of tuberculin; $TCID_{50}$, median tissue culture infective dose.

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plasmids (5). Screening and culturing the recombinant clones and Western blotting of the culture supernatants were carried out as described (6). The rBCG possessing pIJM-V3-19X was named rBCG-V3J1. Anti-HIV-IIIB gp120N (DuPont/NEN) and anti-HIV_{MN} V3 monoclonal antibodies (mAbs) 50.1 (Repligen) and μ5.5 (Dr. Y. Eda, Chemoserotherapeutic Research Institute, Kumamoto, Japan; §§) were used in this study.

Preparation of Clinical HIV Isolates and Sequencing of Their V3 Loops. Viral stocks of HIV-1 clinical isolates were prepared by coculturing phytohemagglutinin (PHA)-activated human peripheral blood mononuclear cells (PBMCs) from both HIV-seropositive and normal individuals, as described by Gorny *et al.* (7). Two Japanese hemophiliac patients (ages 46 and 48 years) seropositive for HIV-1 were selected. HIV infection was detected by routine anti-HIV antibody ELISA and Western blotting and confirmed by virus isolation at the National Institute of Health, Tokyo. The cell-free supernatant was stored at −130°C until used as source of virus. The supernatant virions were precipitated, and RNA was extracted and reverse-transcribed into DNA by using primer OD3 (nt 7345–7369, 5′-AAATTCCCCTCCACAATTAAAACTG-3′). The DNA of the HIV V3 domain was amplified by nested PCR by using the following outer and inner sets of primers, respectively: OA3 (nt 6962–6986; 5′-TGTACACATGGAAT-TAGGCCAGTAG-3′) and OD3 and EB2 (nt 6989–7009 plus a *Bam*HI site; 5′-GCCGGATCCTCAACTCAACTGCTGT-TAAAT-3′) and EC2 (nt 7314–7336 plus a *Pst* I site and stop codon; 5′-GCTCTGCAGTCAAATTTCTGGGTCCCCTC-CTGAGG-3′). PCR products were digested with *Bam*HI and *Pst* I, cloned, and sequenced with M13 primer by the 373A automated DNA sequencer (Applied Biosystems).

§§D'Souza, P., Third International Workshop Antibody Serological Project, March 29, 1992 Keystone, CO, pp. 25–26.

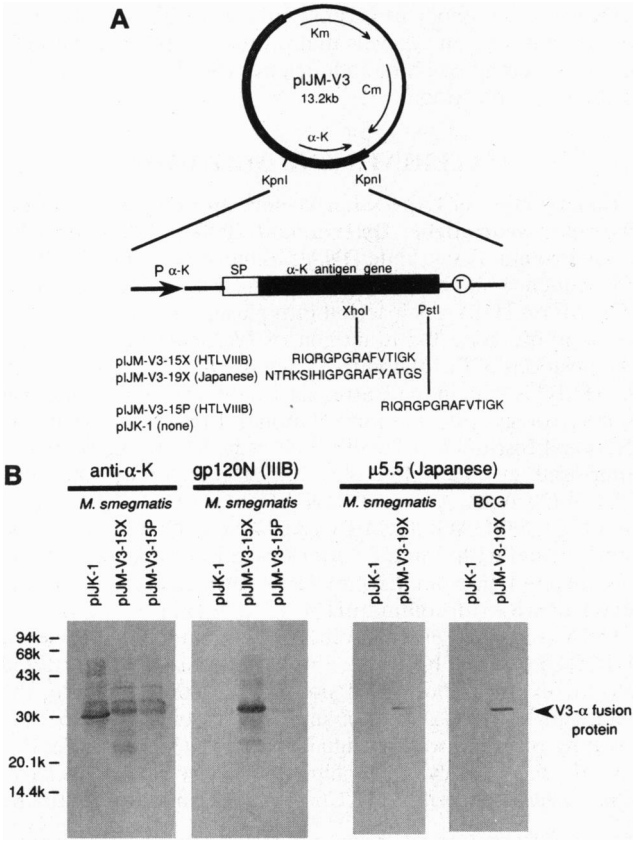

FIG. 1. Vector construction and expression of the PND–α-antigen chimeric protein. (*A*) Construction of pIJM-V3 vector. DNA fragments encoding the PND of the HIV V3 region were inserted into the *Xho* I or *Pst* I restriction site in the gene for α-antigen in plasmid pIJK-1. α-K, Km, and Cm indicate the α-antigen gene and genes for resistance to kanamycin and chloramphenicol, respectively. (*B*) Detection of the PND-α antigen chimeric protein in the culture supernatants by Western blotting.

Table 1. Induction of HIV-specific DTH by inoculation of guinea pigs with rBCG-V3J1

Group	Inoculum	Antigen for challenge	Concentration of antigen, μg/site	Local DTH reaction, mm/site
1	rBCG-V3J1	MPB64-V3J1	10	19 × 19
	None	MPB64-V3J1	10	0 × 0
2	rBCG-V3J1	α-Antigen-V3J1	10	7 × 7
	None	α-Antigen-V3J1	10	0 × 0
3	rBCG-V3J1	KLH-V3J1	10	21 × 21
	None	KLH-V3J1	10	0 × 0
4	rBCG-V3J1	PPD	0.2	19 × 19
	None	PPD	0.2	0 × 0
5	rBCG-V3J1	MPB64	0.2	18 × 18
	None	MPB64	0.2	0 × 0
6	rBCG-V3J1	KLH	10	0 × 0
	None	KLH	10	0 × 0
7	rBCG-V3J1	PBS	—	0 × 0
	None	PBS	—	0 × 0

Hartley strain guinea pigs inoculated with 5 mg of rBCG-V3J1 subcutaneously. Six weeks later, these animals, together with untreated animals, were skin tested by intradermal injection of PPD, KLH-V3J1, KLH, MPB64-V3J1, MPB64, or α-antigen-V3J1. Local DTH skin reactions were measured 24 h after challenge with various antigens. The results are expressed as mean values for eight guinea pigs in each group. PBS, phosphate-buffered saline.

Induction of DTH Skin Reactions by HIV V3 Antigens in Guinea Pigs Injected with rBCG-V3J1. A total of 5 mg (approximately 1×10^8 bacilli) of rBCG-V3J1 were injected subcutaneously into the guinea pigs that were skin-tested six weeks later by intradermal injection of purified protein derivative of tuberculin (PPD, Japan BCG, Tokyo), keyhole limpet hemocyanin (KLH)-conjugated HIV PND (KLH-V3J1), MPB64 protein (8); MPB64-V3J1, or α-antigen-V3J1. The PND peptide specific to the Japanese consensus sequence of HIV was synthesized and conjugated with maleimide-activated KLH, MPB64, or α-antigen. The sequences of the synthesized peptides were confirmed by a protein sequencer.

Table 2. Systemic passive transfer of DTH skin reactions to HIV V3J1 antigens

Group	Target antigen	Origin of spleen cells	Local DTH reaction: Diameter, mm	Local DTH reaction: Thickness, mm
1	PPD	rBCG-V3J1 inoculated guinea pigs	22 × 22	1
2	KLH-V3J1	rBCG-V3J1 inoculated guinea pigs	25 × 25	1
3	KLH	rBCG-V3J1 inoculated guinea pigs	0 × 0	0
4	PPD	Normal guinea pigs	0 × 0	0
5	KLH-V3J1	Normal guinea pigs	0 × 0	0
6	KLH	Normal guinea pigs	0 × 0	0
7	PBS	rBCG-V3J1 inoculated guinea pigs	0 × 0	0

Normal guinea pigs were injected with 100 μg of PPD, KLH-V3J1, KLH, or PBS subcutaneously followed by intravenous injections of spleen cells from rBCG-V3J1-vaccinated guinea pigs or normal guinea pigs. After 24 h, skin reactions were measured. The thickness of the skin reaction site is expressed as the difference from that of normal skin.

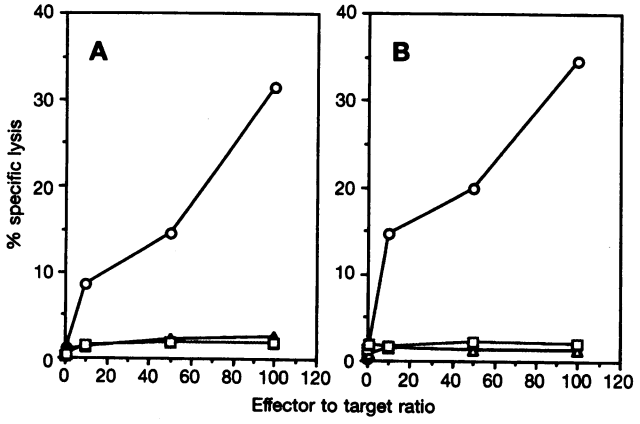

FIG. 2. Specific cytolysis of the PND peptide of the Japanese consensus sequence of HIV-1. (*A*) Cytolytic activity was measured against P815 target cells pulsed or not pulsed with the PND peptide. The restimulated spleen cells from rBCG-V3J1- or rBCG-α-antigen-inoculated mice (□) were incubated with ^{51}Cr-labeled target cells either pretreated (○) with the synthetic V3 peptide or untreated (△). (*B*) Cytolysis is restricted to the major histocompatibility complex class I. Cytolytic activity was measured against P815 (○), BW5147 (△), or S49 (□) cells.

Generation of V3-Specific CTLs. Twenty mice were subcutaneously inoculated with 0.1 mg (approximately 3×10^6 bacilli) of rBCG-V3J1 or rBCG-α-antigen. Spleen cells were isolated from the mice 3 weeks later and stimulated with 10 μg of KLH-V3J1-conjugated protein per ml for 7 days. The restimulated spleen cells were incubated with ^{51}Cr-labeled P815 cells, S49 cells, or BW5147 cells as target cells for 4 h. The target cells had been treated with ^{51}Cr for 60 min, then pulsed with 10 μg of the synthetic V3 peptide per ml for 60 min. The percent of specific ^{51}Cr release was calculated as described by others (9).

Serum Anti-PND Antibody Titration by HIV V3 Peptide-Based ELISA. The wells of 96-well plates were coated with synthetic peptides conjugated with bovine serum albumin (5 μg/ml) at 4°C for 12 h. After the wells of the coated plate were blocked with 2% (wt/vol) nonfat dry milk/0.5% bovine serum albumin/0.02% sodium azide, 100 μl of serial 1:100 dilutions of test serum was added, and the plates were incubated for 2 h at room temperature. After the plates were washed, 100 μl of alkaline phosphatase-conjugated rabbit anti-guinea pig IgG (Zymed) was added. After the wells were washed, *p*-nitrophenyl phosphate (GIBCO/BRL) was added, and absorbance was read with an ELISA reader at 414 nm. The cutoff value of the assay was determined by the mean value of the sera from guinea pigs injected with rBCG-α-antigen plus four SD. The fusion peptide of HIV-1 gp41 (EQELLELDKWASLWNWF) (10) was used as control peptide.

PBMC-Based Virus Neutralization Assay. Serum antibodies were purified from whole sera of 20 immunized guinea pigs inoculated with rBCG-V3J1 or rBCG-α-antigen by using protein A-Sepharose (Pharmacia). Preimmune and normal guinea pig IgG were also purified. The diluted serum antibodies were incubated with 100 medium tissue culture infective dose ($TCID_{50}$) units of HIV_{MN} (H9/HTLV-IIIMN, AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD) for 60 min at 37°C, and the mixture was shaken with 1×10^6 PHA-activated PBMCs for 60 min in a 37°C water bath. After being washed, the cells were cultured in the presence of recombinant human interleukin 2 (40 units/ml, Shionogi, Osaka) for 7 days. The amount of HIV was measured by a p24 antigen ELISA (Dinabot, Tokyo).

The *in vitro* neutralization activity of the immune IgG against primary field isolates from Japanese hemophiliac patients was also determined by using 100 $TCID_{50}$ of the stock virus, as described by Gorny *et al.* (7), and expressed as percent inhibition of p24 antigen production in the culture supernatants compared with that in the cultures to which preimmune serum IgG was added. Virus stocks were titrated on the PHA-activated normal PBMCs, and the $TCID_{50}$ of each virus was determined.

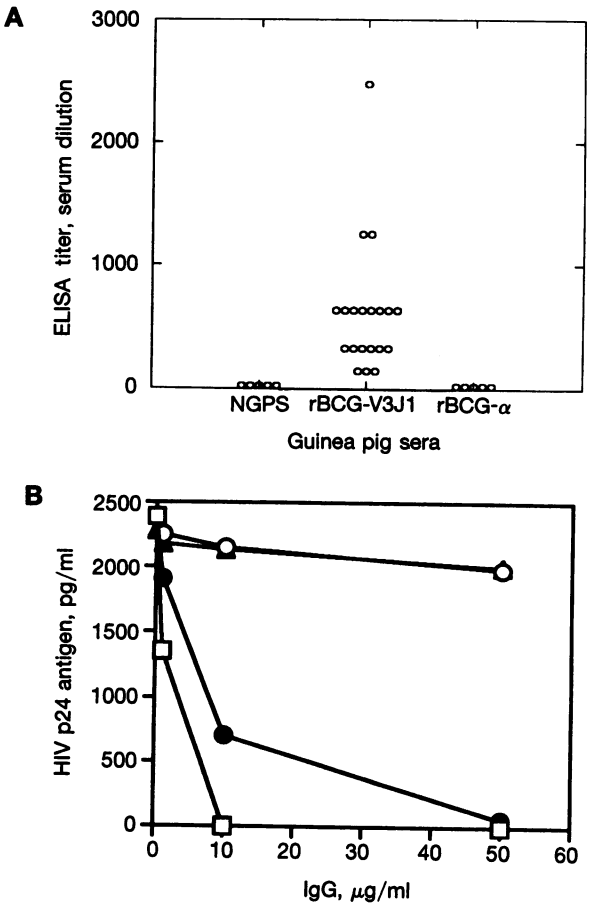

FIG. 3. Induction of humoral immunity by rBCG. (*A*) Serum anti-PND antibody titers determined by HIV V3J1 peptide-based ELISA. Each circle indicates the PND titer of the serum from one animal. NGPS, normal guinea pig sera; rBCG-V3J1, serum from guinea pigs inoculated with rBCG-V3J1 6 weeks earlier; and rBCG-α sera from guinea pigs inoculated with rBCG-α-antigen 6 weeks earlier. The results are expressed as the mean of four different assays. (*B*) *In vitro* neutralization of HIV_{MN} by anti-rBCG-V3J1 antibodies. Virus neutralization activity of serum antibody purified from the 20 guinea pigs inoculated with rBCG-V3J1 (●), from 20 preimmune guinea pigs (○), or from 20 guinea pigs inoculated with rBCG-α-antigen (▲) and of mAb μ5.5 (□). The results are expressed as the mean of four different assays.

Transfer of the Anti-rBCG-V3J1 Antibody to SCID/hu and SCID/PBL Mice and Protection Experiments Against Challenge. SCID mice were constructed by transplanting of $2 \times 2\text{-mm}^3$ blocks of trimmed fetal thymus and liver tissues at 20 weeks of gestation under the left renal capsule (11). After 3 months, the Thy/Liv SCID/hu mice were divided into two groups; one was inoculated first with 10 mg of purified immune IgG, which was followed by intravenous inoculation of 1000 $TCID_{50}$ of HIV_{MN}, and the other was inoculated first with 10 mg of purified normal IgG and then with the same amount of virus. Three weeks later, thymic cells were isolated and cocultured with PHA-stimulated normal human PBMCs for 7 days. SCID/PBL mice were established by injecting them intraperitoneally with 2.5×10^7 cells of Epstein–Barr virus-free PBMCs (12). The mice were then treated similarly as the SCID/hu mice. Seven days later, cells isolated by peritoneal

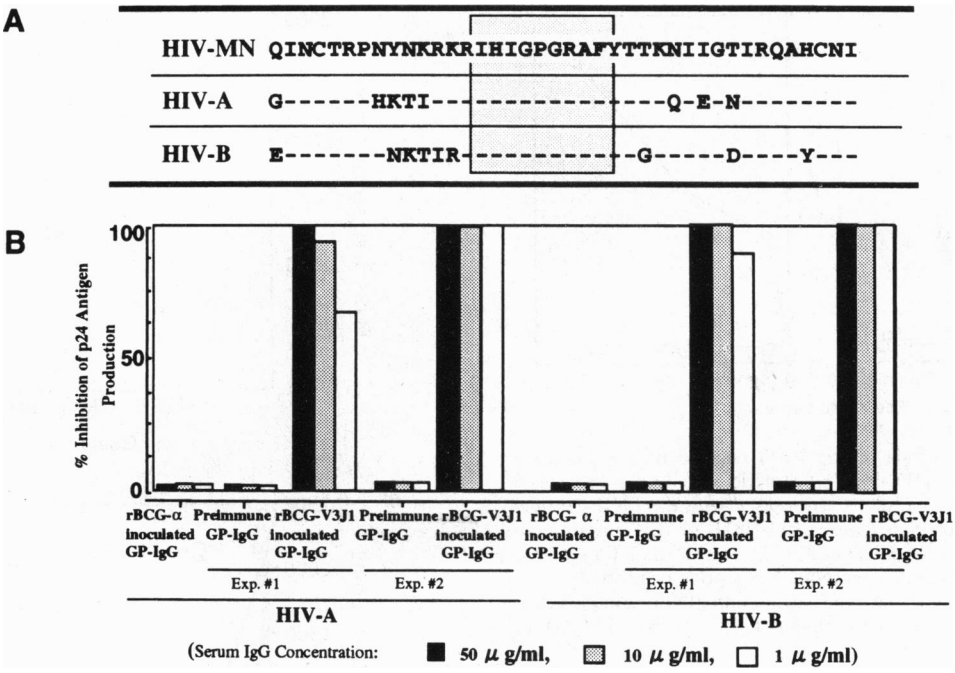

FIG. 4. Neutralization of primary field isolates of HIV-1 by serum IgG from guinea pigs inoculated with rBCG-V3J1. (*A*) The V3 sequence of the isolates from two Japanese hemophiliac patients seropositive for HIV-1 (HIV_A and HIV_B) are aligned with that of HIV_{MN}. Amino acids in the isolates that are identical to the HIV_{MN} sequence are indicated by a dash. The neutralization motifs of the V3 loop in those sequences are enclosed in the shaded rectangle. (*B*) The purified serum IgG from the preimmune guinea pigs, from the rBCG-V3J1-inoculated guinea pigs, and from the rBCG-α-antigen-inoculated guinea pigs as described in the legend to Fig. 3 were used as samples in experiment (Exp. 1). Similarly another 20 guinea pigs were inoculated with 5 mg of rBCG-V3J1, and IgG was purified from the sera and used in Exp. 2. The *in vitro* neutralization activity of the serum IgG samples was determined by using the virus. HIV concentration produced in cultures of PHA-stimulated PBMCs newly infected with HIV_A was 1950 ± 530 pg/ml and in cultures of similar cells infected with HIV_B, the concentration was 2325 ± 615 pg/ml. The results are expressed as the mean of four different assays. GP, guinea pig; rBCG-α, rBCG-α-antigen.

lavage and PBMCs were examined for propagation of HIV-1 in the culture supernatants.

RESULTS

Construction of rBCG-V3J1 That Releases Soluble HIV-1 Chimeric Protein. From hydropathy analysis (5) and B-cell epitope mapping (data not shown), two candidate regions corresponding to the *Xho* I and the *Pst* I restriction sites in the α-antigen gene were selected for insertion of the HIV-IIIB V3 epitope DNA (Fig. 1*A*). To determine the antigenicity of V3–α-antigen chimeric proteins, we used *M. smegmatis* as a host instead of the slow-growing BCG and analyzed the secreted chimeric proteins by Western blot assay (Fig. 1*B*). The chimeric protein secreted by the recombinant *M. smegmatis* clone harboring pIJM-V3-15X had much higher reactivity with the neutralizing mAb (gp120N) than that produced by clones harboring pIJM-V3-15P; therefore, we used the *Xho* I restriction site when inserting the DNA of a 19-mer PND sequence of Japanese consensus HIV. The secretion vector pIJM-V3-19X was introduced into BCG. The rBCG clone secreted a chimeric protein that reacted very strongly with anti-HIV neutralizing μ5.5 (Fig. 1*B*) and 50.1 (data not shown) mAbs.

Table 3. Protection of Thy/Liv SCID/hu and SCID/PBL mice against HIV_{MN} challenge by transfer of guinea pig anti-rBCG-V3J1 antibody

Target cell source	HIV p24 antigen concentration, pg/ml	
	Mice injected with preimmune IgG followed by HIV_{MN}	Mice injected with immune IgG followed by HIV_{MN}
Thy/Liv SCID/hu mice		
Thymic cells	395 ± 180	<30
PBS	<30	<30
SCID/PBL mice		
Peritoneal cells	90 ± 35	<30
PBMCs	95 ± 35	<30

The protective effect of serum antibody from rBCG-V3J1-inoculated guinea pigs was studied as described in *Materials and Methods.*

HIV-PND-Specific Cellular Immune Responses in Guinea Pigs and Mice. DTH to the V3 19-mer peptide of Japanese consensus HIV was studied by using guinea pigs inoculated with rBCG-V3J1 bacilli (Table 1). A control group of animals was left untreated. The DTH responses to KLH-V3J1 6 weeks after inoculation were strongly positive in the animals challenged with PPD, MPB64, MPB64-V3J1 conjugate, or α-antigen-V3J1 conjugate. In contrast, no significant response was observed in control animals or animals challenged with KLH alone (Table 1). The DTH to KLH-V3J1 24 h after the cell transfer was as strongly positive as the transferred PPD response (Table 2). No significant responses were observed in animals receiving spleen cells from normal guinea pigs.

The PND-specific CTLs were clearly induced against syngeneic P815 cells pulsed with the PND peptide at effector-to-target ratios from 100:1 to 10:1 in the mice inoculated with rBCG-V3J1. In contrast, cytolytic activity was not seen in the animals inoculated with the rBCG-α-antigen (Fig. 2*A*). The reactivity was restricted to the class I major histocompatibility complex, $H\text{-}2^d$, because cytolytic activity against allogeneic cells (S49 cells or BW5147 cells) was not demonstrated (Fig. 2*B*).

HIV-PND-Specific Antibody Responses in Guinea Pigs. All 20 guinea pigs exhibited PND-binding antibody activity (serum dilutions from 1:160 to 1:2560) by HIV PND ELISA as late as 6–10 weeks after a single inoculation of rBCG-V3J1 (Fig. 3*A*). No responses to HIV PND were seen with the sera from guinea

pigs immunized by rBCG expressing α-antigen alone. Similar negative results were obtained by the ELISA experiments in which a fusion peptide of HIV gp41 conjugated with bovine serum albumin was used (data not shown).

Serum antibodies purified from the 20 guinea pigs inoculated with rBCG-V3J1 were used in a PBMC-based virus neutralization assay with PBMC-passaged HIV_{MN} (Fig. 3*B*). Measurements of reduction of infectivity showed that the antibodies strongly neutralized the laboratory strain of HIV-1; the 50% suppressive doses of serum antibody and μ5.5 mAb were approximately 7.8 and 3.6 μg of IgG per ml, respectively. In contrast, preimmune IgG purified from the 20 guinea pigs or purified serum antibodies from 20 guinea pigs immunized with the rBCG-α-antigen had no effect on HIV p24 concentration in this assay.

Neutralization of Primary HIV-1 Isolates by Antibodies from Guinea Pigs Immunized with rBCG-V3J1. Two primary clinical isolates, HIV_A and HIV_B, that expressed IHIGPGRAFY at the core sequence of the HIV PND were used as the virus source (Fig. 4*A*). Both HIV_A and HIV_B were strongly neutralized by the immune serum IgG that was shown to neutralize HIV_{MN} in Fig. 3 (Exp. 1, Fig. 4*B*). The potency of the neutralizing action by the immune serum IgG was confirmed by using the IgG purified from immune sera of another 20 guinea pigs inoculated with rBCG-V3J1 in a different experiment (Exp. 2, Fig. 4*B*). The neutralization endpoint at ≥90% was from 1 to 10 μg/ml in the immune serum IgG of guinea pigs inoculated with rBCG-V3J1. However, preimmune IgG or immune serum IgG from guinea pigs inoculated with rBCG-α-antigen showed no activity.

Protection Against HIV-1 After Transfer of anti-rBCG-V3J1 Serum Antibody to SCID/hu or SCID/PBL Mice. All three Thy/Liv SCID/hu mice inoculated with normal guinea pig IgG followed by HIV_{MN} showed significant levels of p24 antigen production in the virus assay described in the legend to Table 3. HIV infection was successfully inhibited in the SCID/hu mice injected with the immune serum antibody before inoculation with the virus (Table 3). HIV infection was also prevented when the serum antibody from the immune guinea pigs inoculated with rBCG-V3J1 was injected into SCID/PBL mice before the HIV_{MN} infection.

DISCUSSION

Both humoral and cell-mediated responses to the HIV PND obtained here suggest that the rBCG-V3J1 is useful for vaccination strategies favoring the induction of stable immunity that could suppress subsequent HIV infection. Recently, Matthews (13) reported that while the vaccinee sera raised by envelope subunit immunogens neutralized appropriate laboratory strains, the same sera failed to neutralize clinical isolates in a PBMC-based virus neutralization assay. In contrast, anti-V3 mAb 447-52D was found to neutralize clinical primary isolates that expressed a GPGR sequence at the center of the HIV V3 domain (14). Further, anti-V2 mAb also neutralized primary but not laboratory isolates of HIV-1 (7). The implication of these observations is that the antibody can neutralize a primary clinical isolate of HIV-1 that expresses the binding sequence and conformation dependence, if the antibody is appropriate. Interestingly, we found that serum antibodies from rBCG-V3J1-inoculated guinea pigs showed neutralizing activity against primary clinical isolates of HIV-1. Since the anti-PND mAb 447-52D can neutralize not only laboratory isolates but also clinical isolates that express a GPXR sequence at the center of the V3 domain (14), the neutralizing antibody of a guinea pig induced by inoculation of rBCG-V3J1 might have antiviral characteristics similar to the 447-52D antibody. Taken together, these results suggest that antibodies are able to neutralize primary HIV-1 isolates when produced appropriately.

The reason why this rBCG vector-based vaccine was able to induce neutralizing antibody against primary clinical isolates is not fully known. One possible explanation is that the chimeric protein produced in the rBCG may conformationally mimic the antigenic site of by the PND region of the field strain. We searched for an appropriate region in the α-antigen sequence in which the candidate sites were located in hydrophilic and antigenic regions. It is possible that the hydrophilic region corresponding to the *Xho* I recognition site was highly exposed in such a way that the inserted V3 region might mimic its original antigenic structure. Further, the chimeric α-antigen might be glycosylated (15), which may favor induction of immune responses in a manner similar to authentic viral antigen. Another report supports the idea that α-antigen is a potent interleukin 2 and interferon γ inducer in cultures of spleen cells from mice infected with BCG (16). Thus, the rBCG vector-based vaccine may induce expression of T_{H1}-enhancing cytokine genes that might be associated with resistance to HIV infection and progression to HIV-induced diseases.

We thank Drs. H. Yoshikura, Department of Bacteriology, Faculty of Medicine, University of Tokyo; and K. Miwa, Ajinomoto, Co., Inc., for their helpful discussions. This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare, Japan, and Japan Health Sciences Foundation Grants 341-5 and 321-2.

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