

# LEGENDplex™ Immune Checkpoint Panels

Multi-Analyte Flow Assay Kits



## HIV Mucosal Vaccine: Nasal Immunization with rBCG-V3J1 Induces a Long Term V3J1 Peptide-Specific Neutralizing Immunity in Th1- and Th2-Deficient Conditions

This information is current as of November 11, 2019.

Takachika Hiroi, Hironobu Goto, Kenji Someya, Manabu Yanagita, Mitsuo Honda, Noboru Yamanaka and Hiroshi Kiyono

*J Immunol* 2001; 167:5862-5867; ;  
doi: 10.4049/jimmunol.167.10.5862  
<http://www.jimmunol.org/content/167/10/5862>

**References** This article **cites 33 articles**, 18 of which you can access for free at:  
<http://www.jimmunol.org/content/167/10/5862.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*\*average*

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2001 by The American Association of  
Immunologists All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# HIV Mucosal Vaccine: Nasal Immunization with rBCG-V3J1 Induces a Long Term V3J1 Peptide-Specific Neutralizing Immunity in Th1- and Th2-Deficient Conditions<sup>1</sup>

Takachika Hiroi,<sup>2\*</sup> Hironobu Goto,<sup>2\*†</sup> Kenji Someya,<sup>‡</sup> Manabu Yanagita,<sup>\*</sup> Mitsuo Honda,<sup>‡</sup> Noboru Yamanaka,<sup>†</sup> and Hiroshi Kiyono<sup>3\*</sup>

In the vaccine strategy against HIV, bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is considered to be one of potential vectors for mucosal delivery of vaccine Ag. We analyzed the induction of the Ag-specific Ab response by nasal immunization with recombinant BCG vector-based vaccine (rBCG-V3J1) that can secrete the V3 principal neutralizing epitope of HIV. Mice were nasally immunized with rBCG-V3J1 (10  $\mu$ g) three times at weekly intervals. Four weeks after the initial immunization, high titers of V3J1-specific IgG Abs were seen in serum. These high levels of HIV-specific serum IgG responses were maintained for >12 mo following nasal immunization without any booster immunization. V3J1-specific IgG-producing cells were detected in mononuclear cells isolated from spleen, nasal cavity, and salivary gland of the nasally vaccinated mice. Nasal rBCG-V3J1 also induced high levels of prolonged HIV-specific serum IgG responses in Th1 (IFN- $\gamma$ <sup>-/-</sup>)- or Th2 (IL-4<sup>-/-</sup>)-immunodeficient mice. Further, IgG3 was highest among V3 peptide-specific IgG subclass Ab responses in these immunodeficient mice as well as in wild-type mice. In addition, this Ag-specific serum IgG Abs induced by nasal immunization with rBCG-V3J1 possessed the ability to neutralize clinical isolate of HIV in vitro. These results suggested that the nasal rBCG-V3J1 system might be used as a therapeutic vaccine in addition to a prophylaxis vaccine for the control of AIDS. *The Journal of Immunology*, 2001, 167: 5862–5867.

Since the onset of the HIV/AIDS epidemic 15 yr ago, the World Health Organization reported that the virus has infected >47 million people in the world (1). With >2.2 million deaths in 1998, HIV/AIDS has now become the fourth leading cause of mortality, and its impact is going to increase. Over 95% of all cases and 95% of AIDS deaths occur in the developing world, mostly among young adults and increasingly in women (1). Since the mean incubation period from seroconversion to the AIDS is approximately 8–10 yr, these infected individuals have the potential to widely disseminate HIV via sexual contact before they become aware of their infection. To control HIV transmission through the epithelium of reproductive tissue, an obvious consideration would be the development of a mucosal vaccine to provide a first line of defense against the invasion of HIV. To this end, mucosal immunization has been shown to induce Ag-specific immune responses in both mucosal and other systemic compartments of the immune system.

Despite intensive debate about whether CTL and/or neutralizing Abs are important for the control of HIV infection, virus-neutralizing Abs are still considered one of the major effector mechanisms against HIV (2). One goal of vaccination should therefore be to induce cross-reactive Abs that can neutralize different strains of the virus for the reduction of the initial virus load while a type-specific host immune response is being activated. A principal neutralizing determinant has been identified in the V3 loop of gp120 (3). Of course, cell-mediated immunity including the induction of CTL and Th1-type responses is also effective against HIV, and the other important goal of vaccination should be to derive cross-reactive CTL, which can rapidly remove virus-infected cells.

The tuberculosis vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)<sup>4</sup> is a widely used vaccine with a low rate of serious complications, especially the Tokyo strain (4). This vaccine has been shown to possess a strong systemic and mucosal adjuvant activity, which can induce both humoral and cell-mediated immune responses (5–8). In previous study a recombinant BCG (rBCG) vector-based vaccine that contained the V3 principal neutralizing, but not a classical, CTL epitope of HIV induced Ag-specific immune responses to the epitope and prevented the viral infection following systemic immunization (5). By using for targeting the sequence of HIV-1 with which most Japanese researchers concur, a chimeric V3 loop protein secretion vector was constructed by selecting an appropriate insertion site of a carrier protein and established the principal neutralizing determinant-peptide secretion system in BCG Tokyo strain (rBCG-expressing

\*Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; <sup>†</sup>Department of Otolaryngology and Head and Neck Surgery, Wakayama Medical University School of Medicine, Wakayama, Japan; and <sup>‡</sup>AIDS Research Center, National Institute of Infectious Disease, Tokyo, Japan

Received for publication March 12, 2001. Accepted for publication September 18, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported in part by grants and contracts from the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan, the Ministry of Education, Science, Sports, and Culture of Japan, and the Ministry of Health and Welfare of Japan.

<sup>2</sup> T.H. and H.G. contributed equally to this work.

<sup>3</sup> Address correspondence and reprint requests to Dr. Hiroshi Kiyono, Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail address: kiyono@biken.osaka-u.ac.jp

<sup>4</sup> Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; CT, cholera toxin; i-LP, intestinal lamina propria; KLH, keyhole limpet hemocyanin; NALT, nasopharyngeal-associated lymphoreticular tissue; NP, nasal passage; PP, Peyer's patch; rBCG-V3J1, recombinant BCG vector-based vaccine that secretes the V3J1 principal neutralizing epitope of HIV; SMG, submandibular glands; SP, spleen.

V3J1; rBCG-V3J1) (5). When guinea pigs were systemically immunized with rBCG-V3J1, a principal neutralizing determinant-specific delayed-type hypersensitivity skin reaction was induced (5). Further, this rBCG-V3J1 immunization induced HIV-specific cytotoxic T lymphocytes in mice (5). The immune serum of IgG was shown to neutralize primary field isolates of HIV that match the neutralizing sequence motif by a PBMC-based virus neutralization assay (5). These findings suggested that the rBCG system could be considered an effective Ag delivery vehicle for the development of HIV vaccine.

In this study we examined a potential of rBCG-V3J1 for mucosal immunization to induce effective V3J1-specific immunity. To accomplish this goal, an rBCG-based mucosal vaccine was tested in normal as well as Th1 (IFN- $\gamma^{-/-}$ ) and Th2 (IL-4 $^{-/-}$ ) type immunodeficient mice to characterize the quality and quantity of virus-specific immune responses. Further, Abs obtained from these immunized mice were examined for neutralizing activity against HIV, including clinical isolate in vitro.

## Materials and Methods

### Mice

C57BL/6J mice and mice of the C57BL/6J background with IFN- $\gamma$  and IL-4 deficiency (IFN- $\gamma^{-/-}$  and IL-4 $^{-/-}$ ) between 8 and 10 wk of age were used in the experiments and purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in barrier-protected animal facilities under pathogen-free conditions using ventilated microisolator cage in the experimental animal facility at the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

### Immunization and sampling schedule

Several groups ( $n = 4$ –8/group) of mice were immunized with rBCG-V3J1 by the nasal (10  $\mu$ g live rBCG;  $\sim 1 \times 10^6$  bacilli), oral (100  $\mu$ g), or systemic (s.c.; 100  $\mu$ g) route. Each group of mice was immunized once per week for 3 consecutive wk, since our preliminary experiments demonstrated that one immunization did not lead to the induction of an Ag-specific Ab response. Serum, saliva, fecal extract, and vaginal wash were collected at weekly intervals for 6–12 mo and monitored for IgG and IgA anti-V3J1-specific Abs.

### Detection of Ag-specific Ab production by ELISA

HIV Ag-specific titers in serum, saliva, fecal extract, and vaginal wash were determined by ELISA using modified methods as described previously (9). ELISA plates were coated with 100  $\mu$ l 2  $\mu$ g/ml V3J1-conjugated keyhole limpet hemocyanin (KLH) protein (V3J1-KLH) or KLH alone resuspended in 0.1 M carbonate buffer. The plates were then incubated with blocking solution (Block Ace, Dainihon, Osaka, Japan). Dilutions of saliva and fecal extract starting at 1/4 and of serum starting at 1/32 were made with blocking solution, and 50  $\mu$ l of each dilution was added to duplicate wells of Ag-coated plates. After incubation at 37°C for 2 h, the coated plates were washed with PBS-Tween and incubated with 100  $\mu$ l of a 1/4000 PBS dilution of peroxidase-conjugated goat anti-mouse IgG and IgA detection Abs (Southern Biotechnology Associates, Birmingham, AL). After incubation for 2 h, plates were washed with PBS-Tween and incubated with 100  $\mu$ l of a 1/2000 PBS-Tween dilution of streptavidin-HRP (Life Technologies, Gaithersburg, MD). After incubation for 1 h, color was developed with TMB (Wako, Tokyo, Japan), stopped with 0.5 N HCl, and measured by absorbance at 450 nm on an ELISA reader (Lab System, Helsinki, Finland).

### Isolation of mononuclear cells

Mononuclear cells from submandibular glands (SMG), nasal passage (NP), and nasopharyngeal-associated lymphoreticular tissue (NALT), Peyer's patch (PP), intestinal lamina propria (i-LP), and spleen (SP) were isolated as previously described (10–12). In brief, mononuclear cells from NALT and SP were isolated by the mechanical dissociation method using gentle teasing through stainless steel screens. NP, SMG, i-LP, and PP mononuclear cells were isolated by the enzymatic dissociation procedure with collagenase type IV (Sigma, St. Louis, MO).

### Detection of V3 peptide-specific Ab-producing cells by ELISPOT assay

Mononuclear cells were analyzed for Ag-specific Ab production at the single-cell level using Ag and isotype-specific ELISPOT assay as previously described (13). Briefly, 96-well filtration plates with a nitrocellulose base (Millititer HA, Millipore, Bedford, MA) were coated with 2  $\mu$ g V3J1-KLH/well. Single-cell suspensions of mononuclear cells from different tissues were added at varying concentrations. After incubation and washing, detection Abs consisting of 1  $\mu$ g/ml HRP-labeled goat anti-mouse  $\alpha$  or anti-mouse  $\gamma$  (Southern Biotechnology Associates) were then added to the plate. The spots were developed by 3-amino-9-ethylcarbazole (Moss, Pasadena, MD) and counted under a dissecting microscope.

### Analysis of cytokine production by V3 peptide-specific T cells

For analyzing V3J1-specific T cell responses, CD4 $^+$  T cells were isolated from NALT, NP, SMG, PP, and SP as previously described by FACS and MACS (10). Purified CD4 $^+$  T cells (>98%) were suspended in complete medium, cultured at a density of  $1 \times 10^6$  cells/ml in the presence of V3J1-KLH (10  $\mu$ g/ml) along with T cell-depleted and irradiated (3000 rad) splenic feeder cells ( $1 \times 10^6$  cells/ml) in flat-bottom 96-well microculture plates (Costar, Cambridge, MA) (14). Following 3-day incubation, culture supernatants of Ag-stimulated T cells were examined for the production of IFN- $\gamma$ , IL-2, IL-4, IL-5, IL-6, and IL-10 by cytokine-specific ELISA. Ag-specific CD4 $^+$  T cell-derived Th1 and Th2 cytokines were measured by murine cytokine ELISA kits (Amersham, Arlington Heights, IL).

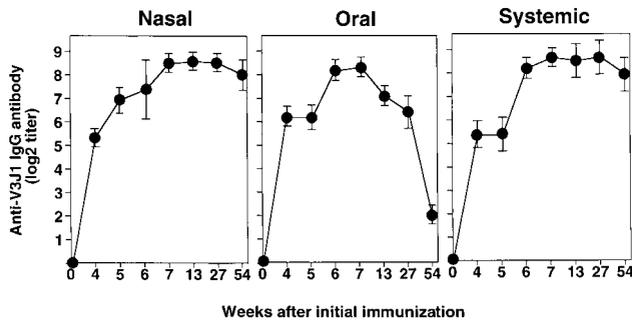
### In vitro HIV neutralization assay

An in vitro neutralizing assay of HIV was performed as previously described (5, 15). Briefly, serum IgG Abs were purified from mice nasally immunized with rBCG-V3J1 using protein A-Sepharose (Pharmacia, Poole, U.K.). Serum IgG was also purified from pre- and nonimmunized mice. The diluted serum Abs were incubated with 100 medium tissue culture infective dose units of HIV-MN (H9/HTLV-III-MN, AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) or HIV-MNp (primary field isolates from Japanese hemophiliac patients; AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan) for 60 min at 37°C, and the mixture was shaken with  $1 \times 10^6$  cocultured PHA-activated human PBMCs for 60 min in a 37°C water bath (5, 15). After washing, the cells were cultured in the presence of recombinant human IL-2 (40 U/ml; Shionogi, Osaka, Japan) for 7 days. Following the incubation, culture supernatants were subjected to a p24 Ag ELISA (Dinabot, Tokyo, Japan) for the measurement of HIV. Data were expressed as the percent inhibition of p24 Ag production in the culture supernatants compared with that in the cultures to which preimmune or nonimmunized serum IgG was added. Virus stocks were titrated on the PHA-activated normal PBMCs, and the 50% tissue culture infective dose of each virus was evaluated as described previously (5, 15).

## Results

### Mucosally administered rBCG-V3J1 induced Ag-specific IgG Abs

High titer values for V3 peptide-specific Abs responses were induced by nasal and oral immunization of rBCG-V3J1 (Fig. 1). The levels of mucosally induced Ag-specific IgG responses were comparable to those induced by the systemic route (Fig. 1). High titers of V3J1-specific IgG Abs were detected 4 wk after initial immunization by all tested administration routes (Fig. 1). It is important to note that high levels of V3 peptide-specific Abs responses were maintained >1 yr (e.g., 54 wk) after initial immunization via nasal and systemic routes compared with oral administration. Further, a lower dose (10  $\mu$ g) of rBCG-V3J1 was required for the induction of an Ag-specific immune response in nasally immunized mice compared with orally immunized mice (Fig. 1). However, V3J1-specific IgA Ab was not detected during the entire period of Ab monitoring in either systemic (e.g., serum) or mucosal secreted (e.g., saliva, fecal extract, and vaginal wash) samples (data not shown). Taken together, these results indicated that nasal vaccination with rBCG-V3J1 was an effective mucosal immunization route for the induction of an Ag-specific systemic immune response. Nasal immunization with rBCG-V3J1 can induce and maintain high levels of HIV-specific IgG Abs responses for >1 yr.



**FIGURE 1.** Induction of V3 peptide-specific IgG Ab by mucosal immunization with rBCG-V3J1. Mice were immunized nasally ( $10 \mu\text{g}$ ), orally ( $100 \mu\text{g}$ ), or s.c. ( $100 \mu\text{g}$ ) with rBCG-V3J1 once a week for 3 consecutive wk. Serum samples were collected and analyzed for V3J1-specific IgG Ab responses at various time intervals by ELISA. The levels of V3J1-specific Ab are expressed as reciprocal end-point titers. When these samples were tested against KLH, the levels of titer in the 7 wk sample were low to undetectable (nasal,  $3.1 \pm 0.5$ ; oral,  $2.2 \pm 0.4$ ; systemic,  $1.6 \pm 0.6$ ). The results represent the values (mean  $\pm$  SEM) for six mice in each experimental group. An identical experiment was repeated on three different occasions with similar results.

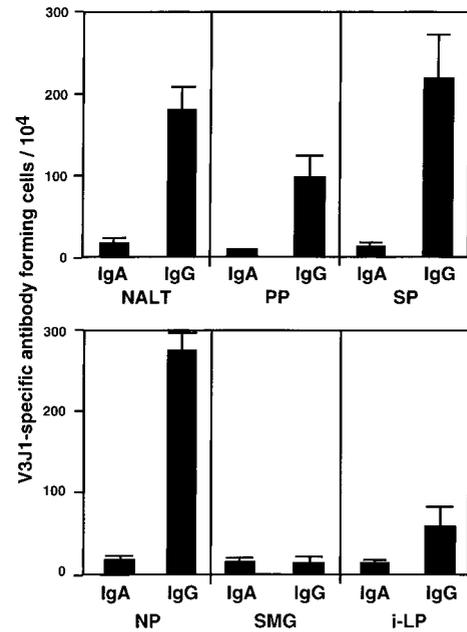
Further, histological analysis revealed no evidences for the development of local pathological change in mice mucosally immunized with rBCG-V3J1. Thus, the detailed analysis of quality and quantity of V3 peptide-specific immune responses was focused on nasal vaccination with rBCG-V3J1 in the rest of this study.

#### Induction of Ag-specific IgG Ab-producing cells by nasal immunization with rBCG-V3J1

Mononuclear cells were isolated from SP and different mucosa-associated tissues (e.g., NALT, NP, SMG, PP, and i-LP) of mice nasally immunized with rBCG-V3J1 to confirm the induction of Ag-specific Ab-forming cells. The isotype and Ag-specific ELISPOT assay revealed the presence of increased numbers of V3 peptide-specific IgG Ab-forming cells in the systemic compartment (e.g., SP; Fig. 2). In addition, Ag-specific IgG Ab-producing cells were detected in several mucosa-associated tissues, including NALT, PP, NP, and i-LP (Fig. 2). To this end, some V3J1-specific IgG Abs were detected in mucosal secretions (e.g., log<sub>2</sub> titer of  $4.7 \pm 0.6$  in fecal extract at 7 wk after immunization). As indicated by the ELISA data (data not shown), however, V3J1-specific IgA Ab-producing cells were not seen in all isolated tissues (Fig. 2). These results showed that nasal immunization with rBCG-V3J1 was capable of inducing Ag-specific IgG-producing cells in both SP and mucosa-associated tissues, including NP, NALT, PP, and i-LP.

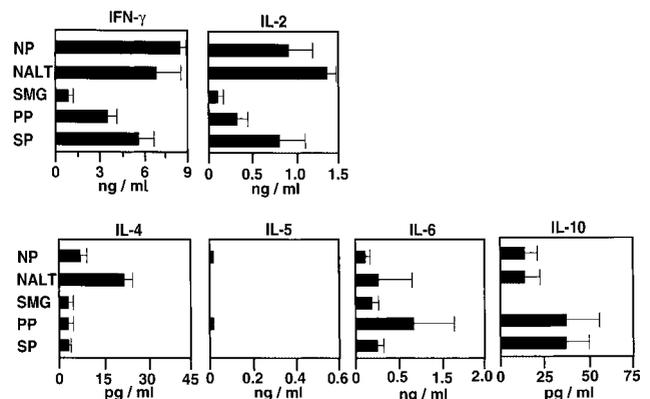
#### Nasal vaccination with rBCG-V3J1 induced Ag-specific Th1-type, but not Th2-type, responses

Inasmuch as high levels of V3J1-specific IgG responses were induced in the systemic immune compartment by nasal vaccination with rBCG-V3J1, it was important to examine the nature of V3J1-specific Th cell responses (e.g., Th1 and Th2 types) induced in these nasally immunized mice. To characterize Ag-specific Th1- and Th2-type responses,  $\text{CD4}^+$  T cells were isolated from SP and mucosa-associated tissues of mice given nasal vaccine and restimulated with V3J1-KLH in vitro. Culture supernatants from V3J1-stimulated  $\text{CD4}^+$  T cells were then examined for the presence of Th1 and Th2 cytokines by ELISA. High levels of Th1 (e.g., IFN- $\gamma$  and IL-2) cytokines were detected in the culture supernatant harvested from in vitro V3J1-stimulated  $\text{CD4}^+$  T cells isolated from



**FIGURE 2.** Nasal vaccination with rBCG-V3J1 induced V3 peptide-specific IgG Ab-producing cells. Mice were nasally immunized with  $10 \mu\text{g}$  rBCG-V3J1 once a week for 3 consecutive wk. After 27 wk mononuclear cells were isolated from SP, NALT, NP, PP, SMG, and i-LP for the analysis of V3J1-specific IgG and IgA Ab-forming cells (AFC) by ELISPOT. Results are the values (mean  $\pm$  SEM) for six mice in each experimental group. An identical experiment was repeated on two different occasions with similar results.

SP and mucosa-associated tissues, such as NP, NALT, and PP (Fig. 3). In contrast, Th2-type cytokines were generally not detected. Taken together, these findings suggested that nasal immunization with rBCG-V3J1 preferentially induced Ag-specific IFN- $\gamma$ - and IL-2-producing Th1-type cells in both systemic and mucosa-associated sites.



**FIGURE 3.** Induction of V3 peptide-specific Th1-type  $\text{CD4}^+$  Th cell responses by nasal immunization with rBCG-V3J1.  $\text{CD4}^+$  T cells ( $1 \times 10^6$  cells/ml) were isolated from SP, NP, NALT, SMG, and PP at 27 wk after nasal immunization with  $10 \mu\text{g}$  rBCG-V3J1. These cells were then cultured with  $10 \mu\text{g}/\text{ml}$  V3J1-conjugated KLH (V3J1-KLH) and APC at  $37^\circ\text{C}$  for 72 h. Culture supernatants were harvested and then analyzed for the production of Th1 and Th2 cytokines by ELISA. Results are the values (mean  $\pm$  SEM) for eight mice in each experimental group. An identical experiment was repeated on two different occasions with similar results.

### V3 peptide-specific IgG Ab responses were induced in both Th1- and Th2-immunodeficient mice by rBCG-V3J1 nasal vaccine

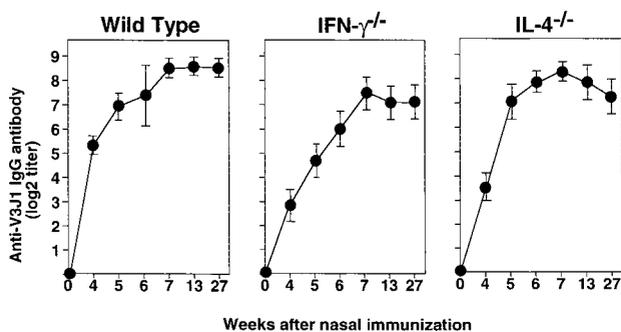
To seek a potential applicability of nasal rBCG-V3J1 for the development of therapeutic vaccine, Th1 (IFN- $\gamma^{-/-}$ )- and Th2 (IL-4 $^{-/-}$ )-deficient mice were nasally immunized with rBCG-V3J1. Increased levels of Ag-specific IgG Abs were induced in these immunodeficient mice (Fig. 4). At levels similar to nasally vaccinated wild-type mice, these high levels of V3 peptide-specific IgG Abs responses were also maintained for at least 27 wk in both IFN- $\gamma^{-/-}$  and IL-4-deficient mice (Fig. 4). When the kinetics of these Ag-specific IgG Ab responses was compared, the induction of high levels of V3 peptide-specific IgG Abs was delayed a few weeks in IFN- $\gamma^{-/-}$  mice compared with IL-4 $^{-/-}$  and wild-type mice (Fig. 4). These results raised the interesting possibility that an rBCG-V3J1 nasal vaccine might be an effective immunization tool to induce V3 peptide-specific immune responses under the different immunodeficient conditions of HIV-infected patients through several progression stages of AIDS.

### Characterization of V3J1-specific IgG subclass Abs in mice nasally administered with rBCG-V3J1

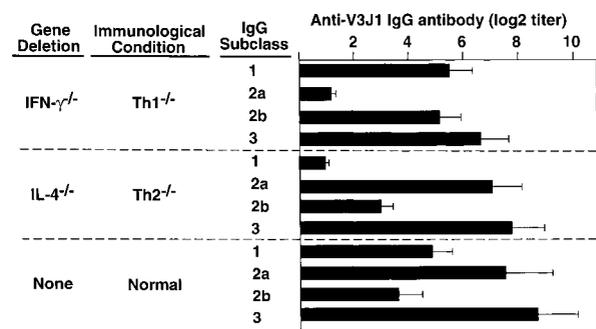
We next examined the IgG subclass of V3 peptide-specific Abs induced by nasal vaccination in these immunodeficient and normal mice. In wild-type mice, the levels of V3J1-specific IgG2a- and IgG3-isotype Abs responses were highest, followed by IgG1 and IgG2b (Fig. 5). As one might expect, Ag-specific IgG1 and IgG2a Abs were not detected in IL-4- and IFN- $\gamma$ -deficient mice, respectively (Fig. 5). However, high levels of V3J1-specific IgG3 Abs were induced in these two immunodeficient mice. These results indicated that high levels of V3 peptide-specific serum IgG3 responses were induced and maintained by rBCG-V3J1 nasal vaccine for at least 0.5–1 yr despite different immunological conditions.

### Neutralization of HIV-1 by V3J1-specific IgG Abs from mice nasally immunized with rBCG-V3J1

For the characterization of virus neutralization activity, a well-characterized laboratory strain of HIV-MN and one primary clinical isolate HIV-MNp, which expressed IHIGPGRIFY at the core sequence of the HIV principal neutralizing determinant (3, 5), were used as the virus source. Nasal rBCG-V3J1-induced serum IgG Abs possessed some neutralizing activity against HIV-MN in wild-type mice (Fig. 6). Further, HIV-MNp was neutralized by the



**FIGURE 4.** rBCG-V3J1 nasal vaccine-induced V3 peptide-specific immune responses in Th1- and Th2-deficient mice. IFN- $\gamma^{-/-}$ , IL-4 $^{-/-}$ , and wild-type mice were nasally immunized with 10  $\mu$ g rBCG-V3J1 once a week for 3 consecutive wk. Serum were collected and analyzed for V3J1-specific IgG Ab at various time intervals by ELISA. Responses are expressed as reciprocal end-point titers. Results are the values (mean  $\pm$  SEM) for six mice in each experimental group. An identical experiment was repeated on two different occasions with similar results.

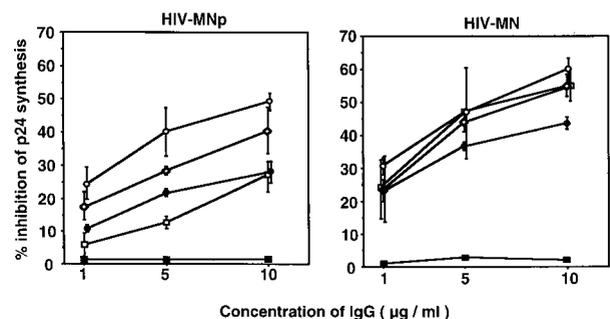


**FIGURE 5.** Characterization of V3 peptide-specific IgG subclass responses in Th1- and Th2-immunodeficient mice nasally vaccinated with rBCG-V3J1. IFN- $\gamma^{-/-}$ , IL-4 $^{-/-}$ , and wild-type mice were nasally immunized with 10  $\mu$ g rBCG-V3J1 once a week for 3 consecutive wk. Twenty-seven weeks after the initial nasal immunization, sera were collected and analyzed for V3J1-specific IgG subclass Ab responses by ELISA. Results are the values (mean  $\pm$  SEM) for four mice in each experimental group. An identical experiment was repeated on two different occasions with similar results.

IgG immune serum (Fig. 6). A similar neutralization activity was noted by the IgG immune serum purified from both IFN- $\gamma$ - and IL-4-deficient mice nasally vaccinated with rBCG-V3J1 (Fig. 6). However, preimmune IgG showed no neutralizing activity (Fig. 6). These results indicate that rBCG-V3J1 nasal vaccine is capable of inducing high V3 peptide-specific IgG Abs with some virus-neutralizing activity. Furthermore, it should be noted that high levels of V3 peptide-specific IgG Abs with a detectable level of neutralizing activity were maintained for a long time (e.g., 7–27 wk) after the nasal immunization.

### Discussion

Considering the present magnitude of the global HIV pandemic and its association with sexual transmission, the development of effective and safe prophylactic HIV vaccines that can provide two layers (e.g., mucosal and systemic sites) of protective immunity is essential for the developing and developed countries. However, one must realize that there are still remaining numerous formidable challenges to accomplish this task. Among those, one obstacle is



**FIGURE 6.** Recombinant BCG-V3J1 nasal vaccine-induced V3 peptide-specific neutralizing Ab responses. In vitro neutralization of HIV-MN and HIV-MNp was performed with a standard p24 release assay. Serum IgG samples were prepared from wild-type mice at 7 wk ( $\diamond$ ) and 27 wk ( $\square$ ) and from immune-deficient mice (IFN- $\gamma^{-/-}$  ( $\circ$ ) and IL-4 $^{-/-}$  ( $\bullet$ )) 6 mo after the initial nasal immunization with 10  $\mu$ g rBCG-V3J1. These immune sera were analyzed for the presence of neutralizing Ab against a clinically isolated HIV-1 strain (HIV-MNp) or a laboratory strain (HIV-MN). As a control, serum samples from preimmunized mice were tested and did not show any Ag-specific neutralization activity ( $\blacksquare$ ). Results are the values (mean  $\pm$  SEM) for four mice in each experimental group.

the lack of clear-cut correlates of protective immunity (16). Despite the gap, a number of observations show the possible existence of such a protective immune response. Long term nonprogressors who remain uninfected and seronegative despite multiple exposures to HIV via the reproductive mucosal epithelium have been identified (17, 18). The presence of V3 HIV-specific CTLs as well as neutralizing Abs has been shown to be correlated with some of nonprogressors (19–21) in addition to the other immunological factors, including CD8 anti-viral factor (18, 22) and chemokines (23). For control of HIV infection, our effort has been focused on the potential application of the mucosal immune system for the development of mucosal vaccine for AIDS. In the present study our findings provided important evidence that a nasal vaccine consisting of rBCG-V3J1 can induce HIV-specific neutralizing IgG Ab responses for a prolonged period (e.g., >1 yr) in mice. Further, the nasal rBCG vector system can be used for therapeutic vaccine in addition to prophylactic purpose, since nasally administered rBCG-V3J1 induced a similar prolonged neutralizing Ab response in both Th1 (IFN- $\gamma^{-/-}$ )- and Th2 (IL-4 $^{-/-}$ )-deficient mice.

An important finding of the present study is a demonstration of the induction of prolonged V3 peptide-specific IgG Ab responses with neutralizing activity by nasal immunization with rBCG-V3J1. To this end, a high titer of Ag-specific IgG Abs was induced and maintained over 1 yr following three doses of nasal immunization without any booster vaccination (Fig. 1). Further, the original BCG vaccination has been shown to induce prolix immunity after minimum doses of immunization (24). Our finding suggested that rBCG carrying the V3J1 gene may intracellularly coincide in selected tissue of nasally immunized mice for continuous delivery of the peptide to immunocompetent cells. In our separate study the current effort is focused on the analysis of a dissemination pathway and determining the source of inhabiting cells and tissues. Our preliminary results suggested that specific message for BCG can be found in NALT and SP 1 wk after final nasal immunization (data not shown). However, we could not yet recover actual BCG from these tissues of mice nasally immunized with rBCG-V3J1. Based on the immunological evidence obtained by the present study, it is plausible to speculate that intracellular rBCG produces V3J1, which continuously stimulates a Th1-type response that, in turn, induces neutralizing IgG Ab responses in nasally immunized mice.

When a subclass of V3 peptide-specific IgG Abs was examined in normal mice nasally immunized with rBCG-V3J1, Ag-specific Ab was found in all subclasses (Fig. 5). However, higher levels of Ag-specific IgG2a and IgG3 Abs were noted compared with IgG1 and IgG2b. According to the profile of V3J1-specific IgG subclass distribution, one would predict that the balance between Th1- and Th2-type responses is shifted toward the former response. Analysis of Th cytokine synthesis by V3J1-stimulated CD4 $^{+}$  T cells from different mucosal and systemic tissues of nasally vaccinated mice further reinforces this view, since a predominant production of Th1 cytokines (e.g., IFN- $\gamma$  and IL-2) was observed in CD4 $^{+}$  T cells isolated from these tissues (Fig. 3). It has been also shown that intracellular micro-organisms such as *Mycobacterium*, *Listeria*, and *Salmonella*, have been shown to preferentially induce Th1-type responses (25–27).

It was interesting to note that increased titers of V3 peptide-specific IgG3 Abs were also induced in addition to Th1-regulated IgG2a and Th2-dependent IgG1 responses (Fig. 5). The latter two subclasses were known to be involved in anti-viral and anti-protein responses, respectively (28). Thus, this Ag-specific IgG subclass Ab could be a key player in the HIV-neutralizing activity induced

by nasal immunization with rBCG-V3J1. In the case of IgG3, this subclass was shown to involve carbohydrate-specific Ab responses (29). At this time we do not know of any explanation or mechanism for the induction of this subclass of Ab by nasally administered rBCG expressing the peptide. Further, the exact contribution of V3 peptide-specific IgG3 in virus-specific immunity is still not known.

It is well known that BCG immunization generally induces a strong Th1-type immune response, including those of high IFN- $\gamma$  synthesis (30, 31). Thus, it was important to examine whether V3J1-specific Th1-type immune responses were induced by nasal vaccination with rBCG-V3J1. As one might expect, high levels of Ag-specific Th1-type CD4 $^{+}$  Th cell-induced IFN- $\gamma$  and IL-2 cytokines were noted (Fig. 3). To this end, high levels of Th1 cell-regulated IgG subclass responses were also induced in these nasally immunized mice (Fig. 5). However, V3J1-specific IgA Ab was not detected through the period of Abs monitoring not only in mucosal secreted samples (saliva, fecal extract, and vaginal wash), but also in serum (data not shown). Because of the preferential induction of Ag-specific Th1-type responses by nasal immunization with rBCG-V3J1, Th2-type immune responses were totally inhibited. Inasmuch as Th2 cell-derived cytokines such as IL-5 and IL-6 have been shown to be essential for the induction of IgA (32), the deficiency of the Th2-type response could be a major contributing factor for the lack of Ag-specific IgA in mice nasally immunized with rBCG-V3J1.

Another important aspect of this study is that nasal vaccination with rBCG-V3J1 is capable of inducing high levels of prolonged Ag-specific neutralizing IgG responses in Th1- or Th2-type immunodeficient mice in addition to wild-type mice for at least 0.5–1 yr (Figs. 1, 4, and 6). In human AIDS, a dichotomy of Th1 and Th2 predominance in HIV-infected individuals has become a central issue of debate. It was originally demonstrated that a switch from Th1- to Th2-type responses occurs during the development of HIV infection (33). However, other studies suggest that a shift from Th1 to Th2 responses does not occur during the progression of HIV infection (34). Furthermore, it was demonstrated that HIV preferentially replicates within CD4 $^{+}$  T cells with the phenotype of Th0 and Th2 cytokine synthesis (35). When we consider the development of a mucosal vaccine, especially for the purpose of a therapeutic vaccine, we must also account for these dysregulated Th1 and Th2 cell profiles in AIDS. Our present finding has provided a potential application of rBCG-V3J1 for the therapeutic mucosal vaccine, since nasal administration of the vaccine construct resulted in the induction of V3J1-specific IgG Abs with neutralizing activity in Th1- and Th2-type immunodeficient mice (Figs. 4–6).

In summary, our findings have presented a new possibility that the mucosal rBCG-V3J1 vaccination system is a useful immunization regimen for not only the prevention of HIV infection but also for the development of therapeutic vaccine. Further, rBCG-V3J1 can induce prolonged V3 peptide-specific IgG Abs with neutralizing activity for more than 0.5–1 yr in both normal and immunodeficient (e.g., IFN- $\gamma^{-/-}$  and IL-4 $^{-/-}$ ) mice. Since rBCG is confirmed for biological safety, and the original Tokyo strain has been already used for a human tuberculosis vaccine, the concept of a nasal rBCG-V3J1 vaccine can be considered for the development of the next generation of AIDS vaccine. However, we have to emphasize that the safety and effectiveness of mucosal rBCG-based HIV vaccine must be continuously and carefully examined both in vivo and in vitro systems before possible application to humans.

## Acknowledgments

We thank Dr. Waka Saito-Inagawa, Kohichi Iwatani, and Noriko Kitagaki as well as the other members of Department of Mucosal Immunology for their technical advice and critical assessment of the study.

## References

- World Health Organization, HIV/AIDS/STI Surveillance. Report on the Global HIV/AIDS Epidemic, December 2000: <http://www.who.int/emc/diseaseses/hiv/>.
- Cohen, O., D. Weissman, and A. S. Fauci. 1999. The immunopathogenesis of HIV infection. In *Fundamental Immunology*, 4th Ed, Chapter 44, W. E. Paul, ed. Raven Press, New York, p. 1455.
- Steimer, K. S., C. J. Scandella, P. V. Skiles, and N. L. Haigwood. 1991. Neutralization of divergent HIV-1 isolates by conformation-dependent human antibodies to Gp120. *Science* 254:105.
- Guerin, N., L. Teulieres, A. Noba, M. Schlumberger, P. Bregere, and P. Chauvin. 1999. Comparison of the safety and immunogenicity of the lyophilized merieux seed and the World Health Organization working reference BCG vaccines in school-aged children in Senegal. *Vaccine* 17:105.
- Honda, M., K. Matsuo, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Kitamura, W. Sugiura, K. Watanabe, Y. Fukushima, S. Haga, et al. 1995. 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. *Proc. Natl. Acad. Sci. USA* 97:10693.
- Langermann, S., S. Palaszynski, A. Sadziane, C. K. Stover, and S. Koenig. 1994. Systemic and mucosal immunity induced by BCG vector expressing outer-surface protein A of *Borrelia burgdorferi*. *Nature* 372:552.
- Kameoka, M., Y. Nishino, K. Matsuo, N. Ohara, T. Kimura, A. Yamazaki, T. Yamada, and K. Ikuta. 1994. Cytotoxic T lymphocyte response in mice induced by a recombinant BCG vaccination which produces an extracellular alpha antigen that fused with the human immunodeficiency virus type 1 envelope immunodominant domain in the V3 loop. *Vaccine* 12:153.
- Lagranderie, M., A. M. Balazuc, B. Gicquel, and M. Gheorghiu. 1997. Oral immunization with recombinant *Mycobacterium bovis* BCG simian immunodeficiency virus nef induces local and systemic cytotoxic T-lymphocyte responses in mice. *J. Virol.* 71:2303.
- Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, M. Yamamoto, K. Fujihashi, F. W. Van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee. 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA* 94:5267.
- Hiroi, T., K. Iwatani, H. Iijima, S. Kodama, M. Yanagita, and H. Kiyono. 1998. Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. *Eur. J. Immunol.* 28:3346.
- Asanuma, H., Y. Inaba, C. Aizawa, T. Kurata, and S. Tamura. 1995. Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. *J. Immunol. Methods* 187:41.
- Mega, J., J. R. McGhee, and H. Kiyono. 1992. Cytokine- and Ig-producing cells in mucosal effector tissues: analysis of IL-5- and IFN- $\gamma$ -producing T cells, T cells receptor expression, and IgA plasma cells from mouse salivary gland-associated tissues. *J. Immunol.* 148:2030.
- Jackson, R. J., K. Fujihashi, J. Xu-Amano, H. Kiyono, C. O. Elson, and J. R. McGhee. 1993. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. *Infect. Immun.* 61:4272.
- Yanagita, M., T. Hiroi, N. Kitagaki, S. Hamada, H. Ito, H. Shimauchi, S. Murakami, H. Okada, and H. Kiyono. 1999. Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. *J. Immunol.* 162:3559.
- Gorny, M. K., A. J. Conley, S. Karwowska, A. Buchbinder, J. Y. Xu, E. A. Emimi, S. Koenig, and S. Zolla-Pazner. 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. *J. Virol.* 66:753.
- Haynes, B. F., G. Pantaleo, and A. S. Fauci. 1996. Toward an understanding of the correlates of protective immunity to HIV infection. *Science* 271:324.
- Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, L. K. Scharager, et al. 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N. Eng. J. Med.* 332:209.
- Cao, Y., L. Qin, L. Zhang, J. Safrin, and D. D. Ho. 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* 332:201.
- Harrer, T., E. Harrer, S. A. Kalams, P. Barbosa, A. Trocha, R. P. Johnson, T. Elbel, and Feinberg. 1996. Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection: breadth and specificity of the response and relation to in vivo viral quasiespecies in a person with prolonged infection and low viral load. *J. Immunol.* 156:2616.
- Klein, M. R., C. A. van Baalen, A. M. Holwerda, S. R. Kerkhof Garde, R. J. Bende, I. P. Keel, J. K. Eeftinck-Schattenkerk, A. D. Osterhaus, H. Schuitemaker, and F. Miedema. 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* 181:1365.
- Pilgrim, A. K., G. Pantaleo, O. J. Cohen, L. M. Fink, and J. T. Zhou. 1997. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. *J. Infect. Dis.* 176:924.
- Scala, E., G. D'Offizi, R. Rosso, O. Turriziani, R. Ferrara, A. M. Mazzone, and G. Antonelli. 1997. C-C chemokines, IL-16, and soluble antiviral factor activity are increased in cloned T cells from subjects with long-term nonprogressive HIV infection. *J. Immunol.* 158:4485.
- Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  as the major HIV-suppressive factors produced by CD8<sup>+</sup> T cells. *Science* 270:1811.
- Ferrari, G., D. D. Kostyu, J. Cox, D. V. Dawson, J. Flores, K. J. Weinhold, and S. Osmanov. 2000. Identification of highly conserved and broadly cross-reactive HIV type 1 cytotoxic T lymphocyte epitopes as candidate immunogens for inclusion in *Mycobacterium bovis* BCG-vectored HIV vaccines *AIDS Res. Hum. Retroviruses* 20:1433.
- Kumar, M., A. K. Behera, H. Matsuse, R. F. Lockey, and S. S. Mohapatra. 1999. A recombinant BCG vaccine generates a Th1-like response and inhibits IgE synthesis in BALB/c mice. *Immunology* 97:515.
- Marielen, M., and P. Yvonne. 1999. Th1 T cell responses to HIV-1 gag protein delivered by a *Listeria monocytogenes* vaccine are similar to those induced by endogenous listerial antigens. *J. Immunol.* 163:1449.
- Yamamoto, M., J. L. Vancott, N. Okahashi, M. Marinaro, H. Kiyono, K. Fujihashi, R. J. Jackson, S. N. Chatfield, H. Bluethmann, and J. R. McGhee. 1996. The role of Th1 and Th2 cells for mucosal IgA responses. *Ann. NY Acad. Sci.* 778:64.
- Coutelier, J. P., J. T. van der Logt, F. W. Heessen, G. Warnier, and J. van Snick. 1987. IgG2a restriction of murine antibodies elicited by viral infections. *J. Exp. Med.* 165:64.
- Perlemutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 121:566.
- Yang, J., and M. Mitsuyama. 1997. An essential role for endogenous interferon- $\gamma$  in the generation of protective T cells against *Mycobacterium bovis* BCG in mice. *Immunology* 91:529.
- O'Donnell, M. A., Y. Luo, X. Chen, A. Szilvasi, S. E. Hunter, and S. K. Clinton. 1999. Role of IL-12 in the induction and potentiation of IFN- $\gamma$  in response to bacillus Calmette-Guérin. *J. Immunol.* 163:4246.
- Beagley, K. W., J. H. Eldridge, F. Lee, H. Kiyono, M. P. Everson, W. J. Koopman, T. Hirano, T. Kishimoto, and J. R. McGhee. 1989. Interleukins and IgA synthesis. human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* 169:2133.
- Clerici, M., F. T. Hakim, D. J. Venzon, S. Blatt, C. W. Hendrix, T. A. Wynn, and G. M. Shearer. 1993. Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J. Clin. Invest.* 91:759.
- Graziosi, C., G. Pantaleo, K. R. Gant, J. P. Fortin, J. F. Demarest, O. J. Cohen, R. P. Sekaly, and A. S. Fauci. 1994. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. *Science* 265:248.
- Maggi, E., M. Mazzetti, A. Ravina, F. Annunziato, M. de Carli, R. Piccinni, M. P. Manetti, M. Carbonari, A. M. Pesce, G. del Prete, et al. 1994. Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science* 265:244.