

Prevention of Encephalomyocarditis Virus-Induced Diabetes by Live Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin in Susceptible Mice

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The D variant of encephalomyocarditis (EMC-D) virus causes diabetes in susceptible mice by direct cytolysis of pancreatic β -cells. cDNA covering the major outer capsid protein (VP1) of the EMC-D virus was cloned into *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). None of the SJL/J mice immunized with live recombinant BCG-VP1 (rBCG-VP1) became diabetic when challenged with the highly diabetogenic EMC-D virus, but the control mice inoculated with normal BCG developed diabetes during the same challenge. VP1-specific antibodies (including neutralizing antibodies) were markedly increased over time and reached the maximum titer at week 10 after a single immunization. The plateau of the titer lasted longer than 4 weeks. Mice and guinea pigs immunized with live rBCG-VP1 showed strong delayed-type hypersensitivity to the VP1 of the EMC-D virus. The preventive immunity still worked effectively 10 months after the primary immunization. At that time, the VP1-specific antibody was almost undetectable in the bloodstream, but a large number of VP1-specific lymphocytes was found in the spleen of the immunized mice. Our results show that live rBCG-VP1 elicits effective humoral and long-lasting cellular immune responses against EMC-D virus infection that results in the prevention of virus-induced diabetes in susceptible mice. *Diabetes* 49:1459-1467, 2000

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ADC, albumin, dextrose, catalase; BCG, bacillus Calmette-Guérin; cfu, colony-forming units; cpm, counts per minute; CTL, cytotoxic T-lymphocyte; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; EMC-D, D variant of encephalomyocarditis; OADC, oleic acid, albumin, dextrose, catalase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pfu, plaque-forming units; PPD, purified protein derivative of tuberculin; rBCG, recombinant bacillus Calmette-Guérin; rVP1, recombinant VP1 protein; SI, stimulation index; TCID₅₀, tissue culture infectious dose.

Type 1 diabetes, one of the most common metabolic disorders, results from the destruction of pancreatic β -cells. Several experiments have shown that viruses as environmental factors can cause diabetes in humans and experimental animals (1-5). The D variant of encephalomyocarditis (EMC-D) virus induces diabetes in more than 90% of susceptible mice not by autoimmunity (6-9) but by direct infection and cytolysis of pancreatic β -cells (4,5). Several reports have shown that the β -cell tropism of the EMC-D virus is strongly related to the major outer capsid protein VP1 (10-13).

Recently, several live recombinant vaccine vehicles are being developed to produce a new generation of vaccines against a broad spectrum of infectious diseases. Among the possible bacterial and viral vaccine vector candidates, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is particularly attractive because of specific characteristics such as low toxicity, adjuvant potential (14), low cost, and long-lasting immunity-inducing capacity (15,16). Recombinant BCG (rBCG) expressing foreign antigens is a promising candidate for the development of future vaccines and has been shown in several experimental models to induce protective immunity against viral, bacterial, or parasitic infections. Stover et al. (17) developed shuttle plasmids that are capable of autonomous replication in *Escherichia coli* and BCG. Since then, many investigators have constructed rBCG as a vaccine candidate against multiple pathogens and have examined its use as a live vaccine (17-23).

This work was initiated to see whether the rBCG system is applicable for the prevention of virus-induced diabetes in the animal model. We have cloned the VP1 gene of the EMC virus into a BCG expression plasmid and transformed it into BCG. SJL/J male mice were immunized with rBCG and then were challenged with the highly diabetogenic EMC-D virus. None of the rBCG-immunized mice became diabetic, whereas control or vector-transformed BCG-immunized mice developed diabetes during the same challenge. The rBCGs were found to express VP1 efficiently in vitro and could induce substantial amounts of neutralizing antibodies and delayed-type hypersensitivity (DTH) to the EMC virus in the immunized animals. The rBCG-induced preventive immunities lasted longer than 10 months in the immunized mice. These long-lasting immunities are likely to be associated more with VP1-specific cellular immunity than with humoral immunity.

RESEARCH DESIGN AND METHODS

Animals. The 5- to 6-week-old BALB/c mice were obtained from the Korea Chemistry Institute. SJL/J mice were purchased from Jackson (Bar Harbor, ME) and then were bred and maintained in the Animal Resource Center at the Korea Research Institute of Bioscience and Bioengineering. Inbred guinea pigs were maintained at the Korea Institute of Tuberculosis (Seoul). All animals were kept under specific pathogen-free conditions at each facility.

Brief description of whole experiments. Whole experiments are schematically illustrated in Fig. 1. The polymerase chain reaction (PCR) fragment of VP1 cDNA of the EMC-D virus was inserted into the pMV261 vector and transformed into the BCG. SJL/J male mice were immunized with the rBCG and then were tested for preventive immunity against the highly diabetogenic EMC-D virus. Serum and urine glucose levels were measured to evaluate diabetes in the challenged mice. Cellular and humoral immune responses were analyzed in the immunized mice for 10 months.

Molecular cloning of EMC-D virus VP1 into BCG. The VP1 region of the EMC-D virus was amplified from the pEDfH cDNA clone (24) by PCR with PCR primers. Sense 5'-ACCCGGGATCCGGGAGTTGAGAATGCT GAG-3' and antisense 5'-GCATTAAGCTTATCCAGCATAAGGACTCC-3' primers containing *Bam*HI and *Hind*III sites (which are underlined), respectively, were designed based on the sequence of the EMC-D virus reported previously (11). The PCR fragment of 831 bp was integrated into *Bam*HI/*Hind*III sites of *E. coli*/*Mycobacterium* shuttle vector pMV261 (17) (MedImmune) and pRSET/B (Invitrogen, San Diego, CA), which were then named pMV261-VP1 and pRSET-VP1, respectively (Fig. 1). pMV-VP1 recombinant plasmid was introduced into *M. bovis* BCG 1173-P2 Pasteur strain by Electroporator II (Invitrogen) as described previously (25). After electroporation, 50 μ l 5 \times Dubos medium was added to the BCG-DNA suspension, and the mixture was incubated for 1 h. Cells were plated on Middlebrook 7H11 agar with oleic acid, albumin, dextrose, catalase (OADC) enrichment (Difco, Detroit, MI) and kanamycin (25 μ g/ml). Kanamycin-resistant colonies were subcultured in Middlebrook 7H9 liquid medium containing albumin, dextrose, catalase (ADC) enrichment (Difco) and kanamycin for 1 week. Recombinant pRSET-VP1 was introduced into *E. coli* BL21(DE3) and then was named pRSET-VP1/BL21(DE3).

VP1 protein expression in rBCG. Recombinant BCG-VP1 (rBCG-VP1) was grown to mid-log phases in 7H9 broth supplemented with 25 μ g/ml kanamycin at 37°C and then rapidly shifted to 45°C. After incubation for 2 h at 45°C, cells were harvested and resuspended in 1/20-vol radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mmol/l Tris, pH 8.0). The cell lysates were analyzed by Western blot hybridization with mouse antiserum obtained from EMC-D-infected SJL/J mice and alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma, St. Louis, MO). Recombinant VP1 protein (rVP1) was purified from the cell lysates of pRSET-VP1/BL21(DE3) culture as described previously (26) using a Ni²⁺-nitrilotriacetic acid agarose resin column (Qiagen, Chatsworth, CA).

Immunization. The 5- to 6-week-old SJL/J male mice were inoculated with heat-induced rBCG-VP1 and control BCG at a concentration of 1×10^7 cells/mouse i.p.

Challenge and protection assay. A total of 4–6 weeks after immunization with rBCG-VP1 or control BCG, SJL/J male mice were challenged with the highly diabetogenic EMC-D virus at a concentration of 10^6 plaque-forming units (pfu)/mouse i.p. Serum and urine glucose levels were examined weekly after the challenge to determine whether the inoculated mice would become diabetic. The mice who were challenged with the EMC-D virus but who maintained their serum and urine glucose levels within the normal range of control mice were recorded as having vaccine-mediated protection from the virus-induced diabetes. Two mice from each group were killed 1 and 2 weeks after challenge, and the pancreases were fixed in 10% buffered formalin phosphate. Paraffin-embedded sections were stained with hematoxylin and eosin, and the mouse islets were examined under a light microscope as described previously (7). Pancreases and sera of the challenged mice were also examined to detect free virus particles after challenge, which were counted by tissue culture infectious dose (TCID₅₀) or plaque assay (24).

Serum and urine glucose level. Urine glucose level was determined semi-quantitatively with the Glucotest (Boehringer Mannheim, Mannheim, Germany). Serum glucose level was measured with the Glucose Assay Kit (Sigma) according to the instruction manual. The serum glucose level of each mouse was expressed by the glucose index, which was calculated from the values of glucose tolerance tests and nonfasting glucose levels as described previously (12). Individual mice were scored as diabetic when their glucose indices were $>(\text{mean} + 5 \text{ SD})$ of control preimmune sera.

VP1-specific antibody and isotyping. In the serum collected from the rBCG-VP1-immunized mice, VP1-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (27) using 0.5 μ g/ml disrupted EMC-D virus for coating and alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody. Antibody

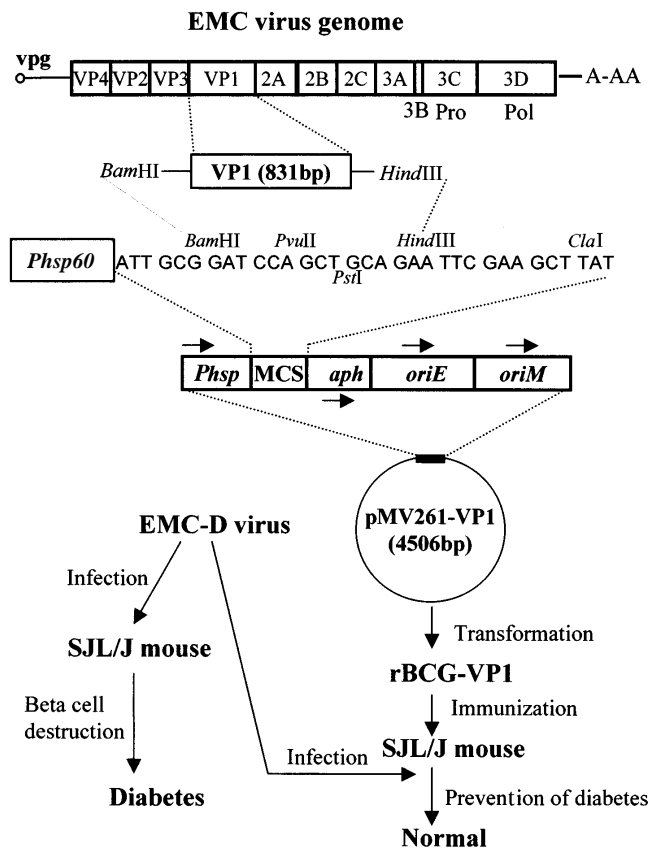


FIG. 1. Schematic illustration of the experimental procedures. A-AA, poly(A) tail; apb, gene conferring kanamycin resistance as a selectable marker; MCS, multiple cloning site; *oriE*, *E. Coli* origin of replication; *oriM*, mycobacterial plasmid origin of replication; *Phsp60*, promoter for heat shock protein; Pol, polymerase; Pro, protease; vpg, virion protein genome.

titer was expressed by the $A_{405 \text{ nm}}$ values or the highest dilution rate yielding absorbency 3 times greater than the background. For isotyping, plates were coated with 1 μ g/ml rVP1 and were incubated with the mouse sera. After washing, goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Pharmingen) were added to each well, respectively, and were incubated for 1 h at room temperature. Immune complexes were detected with a horseradish peroxidase-conjugated rabbit anti-goat antibody (Sigma).

Neutralizing antibody in the serum. Twofold serially diluted serum samples were mixed and incubated with 125 TCID₅₀ U of EMC-D virus at 37°C for 1 h. HeLa cell suspension was added to each well by 1×10^4 cells and then was incubated for an additional 7 days. Cells were stained with 1% crystal violet. The titer of neutralizing antibody was expressed as the maximum reciprocal dilution rate of serum preventing 50% cytopathology. Titers were denoted after deduction of the basal value of preimmune sera.

Genetic stability of the rBCG-VP1 in vivo. Immunized SJL/J male mice were killed 8 weeks after immunization. Spleens were homogenized in phosphate-buffered saline (PBS) with 0.05% Tween 80 (pH 7.4) and were plated on Middlebrook 7H11 agar with OADC enrichment and kanamycin (25 μ g/ml). Kanamycin-resistant colonies were subcultured in Middlebrook 7H9 liquid medium containing ADC enrichment and kanamycin. Lysates from these cultures were tested for VP1 expression by Western blot, and plasmid DNA extracted from the recovered BCG clone was tested for its integrity by PCR with VP1-specific primers.

DTH reaction. Immunized mice and guinea pigs were used for the DTH reaction. The rBCG-immunized mice were tested for DTH responses at 3 different time points starting at 6 weeks after the first immunization as described previously (28) using 1 μ g rVP1 for inoculation into hind footpads. The thickness of footpads was measured 48 h after injection, and the DTH responses were represented as the percentage of increase of footpad swelling at the injection site based on the footpad thickness before injection. Inbred guinea pigs were administered 1×10^8 cells i.p. live rBCG-VP1 and control BCG. A total of 4 weeks later, they were boosted with the same amounts of cells. At 2 weeks

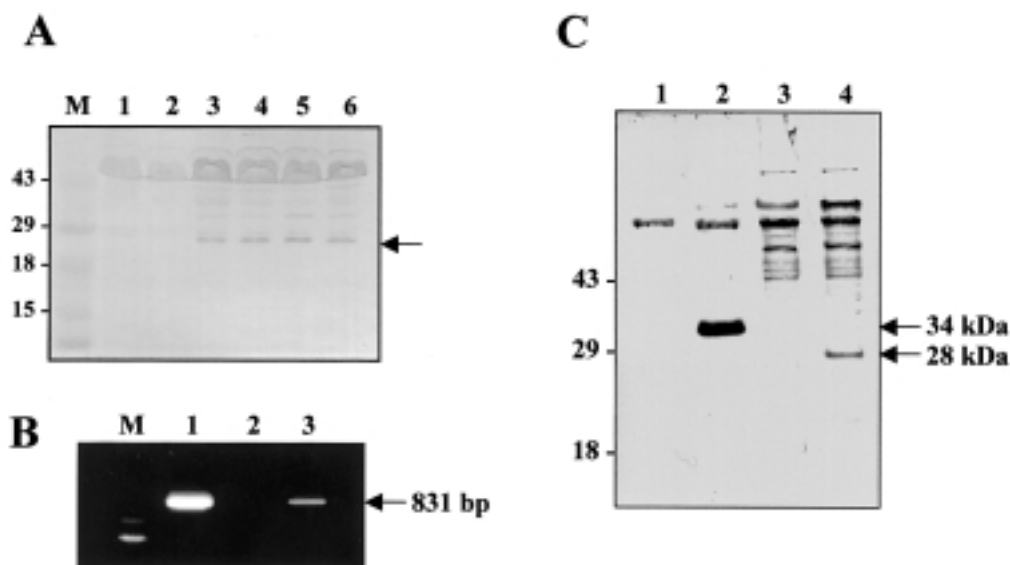


FIG. 2. A: Western blot analysis of rBCG-VP1 lysates with mouse anti-EMC-D antiserum. rBCG-VP1 cultures were heat induced at 45°C for 2 h in the log phase, and then identical amounts of cells were harvested from the culture every week. Each lane shows protein size marker (M), control rBCG-pMV (lane 1), heat-induced control rBCG-pMV (lane 2), and rBCG-VP1 harvested 1 week (lane 3), 2 weeks (lane 4), 3 weeks (lane 5), and 4 weeks (lane 6) after a single heat shock. **B:** PCR amplification of the plasmid extracted from the kanamycin-resistant rBCG that was isolated from the spleens of the immunized mice with VP1-specific primers. Each lane shows 100 bp DNA ladder marker (M), pMV-VP1 positive control (lane 1), pMV261 negative control (lane 2), and plasmid recovered from the rBCG-VP1-immunized mice 8 weeks after immunization (lane 3). **C:** Western blot analysis of rBCG recovered from the immunized mice with anti-EMC-D antiserum. Each lane shows BL21 (DE3) lysate (lane 1), pRSET-VP1/BL21 (DE3) lysate (lane 2), rBCG-pMV (lane 3), and rBCG-VP1 (lane 4).

after boosting, these animals were skin tested by intradermal injection of purified rVP1 protein and purified protein derivative of tuberculin (PPD) (Korea Institute of Tuberculosis). DTH responses were determined 48 h after injection by measuring induration size using the Vernier Digital Caliper (Fisher Scientific, Fair Lawn, NJ).

Enzyme-linked immunospot test. To detect the VP1-specific antibody-producing cells in the rBCG-VP1-immunized mice, we performed the enzyme-linked immunospot (ELISPOT) assay. Mononuclear single-cell suspensions were prepared from spleens of the immunized mice by dissociating the spleens using the Cell Dissociation Sieve-Tissue Grinder Kit (Sigma), were purified by Ficol-Histopaque (Sigma) density-gradient centrifugation, and were resuspended at 2×10^6 cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco/BRL, Grand Island, NY). Cells were incubated in the presence of 2 μ g/ml rVP1 protein for 24 h. Each fraction of the splenocytes (5×10^5 cells/ml) was then added to the nitrocellulose plates (Millipore, Bedford, MA), which were precoated with purified rVP1 protein at 2 μ g/ml in a bicarbonate buffer. The following experimental steps were performed as described previously (29). The number of spots was determined under a microscope.

VP1-specific lymphocyte proliferation assay. Splenocytes were purified as described above. T-cell-enriched (>90%) splenocyte fractions were prepared by depletion of B-cells from the splenocyte suspensions by panning with anti-mouse IgG as previously described (30). Splenocytes from uninfected syngeneic mice were used as a source of antigen-presenting cells. For proliferation assays, panned T-cells (2×10^5 /well) and antigen-presenting cells (2×10^4 cells/well) were cultured in 96-well plates in the presence or absence of affinity-purified rVP1 protein (0.4, 2, and 5 μ g/ml) for 5 days. [3 H]thymidine (DuPont-NEN, Boston, MA) was added to each well (0.5 μ Ci/well) during the last 18 h of culture. Lymphocyte proliferation was expressed by a stimulation index (SI) calculated as counts per minute (cpm) with antigen stimulation/cpm without antigen stimulation.

Statistical analysis. The significant differences between experimental groups were evaluated by Student's *t* test. The data with a *P* value ≤ 0.05 were considered to be significant.

RESULTS

rBCG-VP1 construction, VP1 expression, and its genetic stability in vitro and in vivo. The recombinant plasmid (pMV-VP1) was constructed by joining PCR-amplified VP1

cDNA of the EMC-D virus into the pMV261 vector. pMV-VP1 was introduced into the BCG 1173-P2 Pasteur strain, and the positive clone was named rBCG-VP1. rBCG-VP1 expressed detectable amounts of VP1 protein even by a single heat induction, and the expression was maintained for 4 weeks without further heat induction (Fig. 2A). An average of 1.5×10^3 colony-forming units (cfu) of kanamycin-resistant rBCG was recovered per spleen from the mice 8 weeks after injection of 1×10^6 cfu i.p. Splenic isolates retained the recombinant plasmid containing the VP1 cDNA fragment (Fig. 2B) and expressed VP1 protein (Fig. 2C) when examined after regrowth in vitro. These results indicate that rBCG-VP1 is genetically stable in vivo in the absence of antibiotic selection. Discrepancies in the molecular weight of rVP1 (Fig. 2C, lanes 2 and 4) are because of the differences in nongenic sequences deduced from each plasmid.

rBCG-VP1 immunization protects SJL/J mice from EMC-D-induced diabetes. Each group ($n = 12$) of SJL/J male mice was inoculated with control BCG (rBCG-pMV, BCG containing pMV vector only) and heat-induced rBCG-VP1, respectively. When challenged at week 6 with the highly diabetogenic EMC-D virus, none of the rBCG-VP1-immunized mice ($n = 10$, $P < 0.01$) became diabetic (Table 1). Urine and serum glucose levels of the mice were maintained at normal ranges throughout the period of the experiments (Fig. 3). Neither a symptom of insulinitis in the histological examination nor any detectable amounts of virus could be detected in the pancreases of the rBCG-VP1-immunized mice even a week after challenge (data not shown). Considering the reports that even 100 pfu EMC-D virus is enough to cause diabetes in SJL/J male mice (7), our results suggest that most of the challenged virus particles (10^6 pfu) were effec-

TABLE 1
Prevention of virus-induced diabetes by immunization with rBCG-VP1

Immunization	Challenge with EMC-D virus	Diabetic mice (n)*	Glucose index (mg/dl)
Control BCG (BCG-pMV)	+	8 of 10	387 ± 93
	-	0 of 10	158 ± 15
rBCG-VP1	+	0 of 10	161 ± 16
	-	0 of 10	148 ± 12
PBS only	+	4 of 5	420 ± 87
	-	0 of 5	154 ± 16

Data are means ± SD, unless otherwise indicated. Mice inoculated with 1×10^7 cells/mouse i.p. were challenged with 1×10^6 pfu of the highly diabetogenic EMC-D virus 6 weeks after primary inoculation. *Mice whose glucose indexes were >234 mg/dl (5 SD above the mean glucose index of normal control mice) were scored as diabetic ($P < 0.05$).

tively neutralized within a week by rBCG-VP1-induced protective immunity.

A total of 8 out of 10 rBCG-pMV-immunized mice became diabetic when challenged with the EMC-D virus (Table 1). Urine glucose level was dramatically increased starting between day 4 and day 5 and reached the maximum between day 7 and day 10. The histogram pattern of serum glucose level tested weekly was similar to that of urine glucose in these mice (Fig. 3). Serum glucose level reached the maximum in a week and then was maintained at ~ 400 mg/dl through out the following weeks ($P < 0.05$). Histological examination of the pancreatic section of control BCG-immunized mice showed typical insulinitis with severe β -cell necrosis in most islets as shown previously (9,13,31) even a week after the challenge with the EMC-D virus (data not shown). A week after the challenge, control BCG-immunized mice showed viremia (10^2 – 10^4 pfu/ml) in the blood stream, and a high titer of free virus particles

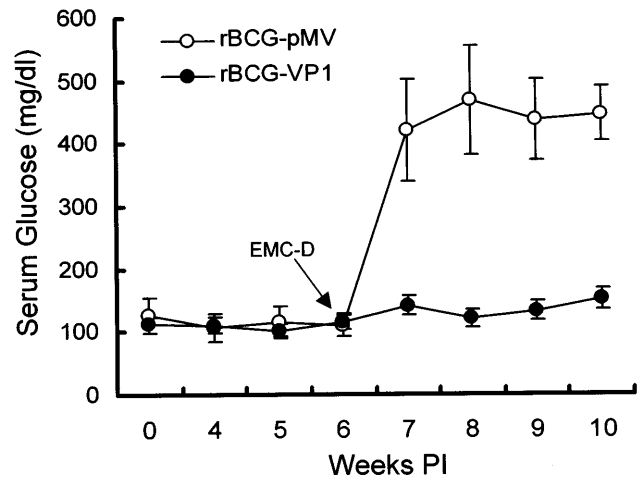


FIG. 3. Mice immunized with rBCG-VP1 were resistant to EMC-D virus-induced diabetes. Two groups of SJL/J male mice ($n = 10$ per group) were inoculated with 1×10^7 cells of control rBCG-pMV and rBCG-VP1, respectively. Six weeks postinfection (PI), mice were challenged with 1×10^6 pfu i.p. of the EMC-D virus. The serum glucose level of each mouse was measured weekly using the Glucose Assay Kit. The arrow indicates the time point of challenge with the highly diabetogenic EMC-D virus. Data are the geometric means ± SD of the serum glucose concentrations of immunized mice at each time point ($P < 0.05$).

(2 – 5×10^5 pfu) was recovered from the pancreas. In the following weeks, however, the viremia in the blood stream and free virus particles in the pancreas were not detected even in the control BCG-immunized mice.

VP1-specific IgG and neutralizing antibody against the EMC-D virus in rBCG-VP1-immunized mice. SJL/J mice ($n = 10$) inoculated with a single dose of rBCG-VP1 (1×10^7 cells/mice) had a marked increase in the VP1-specific antibody ($P < 0.01$ vs. normal control) during the 4–10 weeks after a single inoculation of rBCG-VP1 (Fig. 4A). Reciprocal ELISA

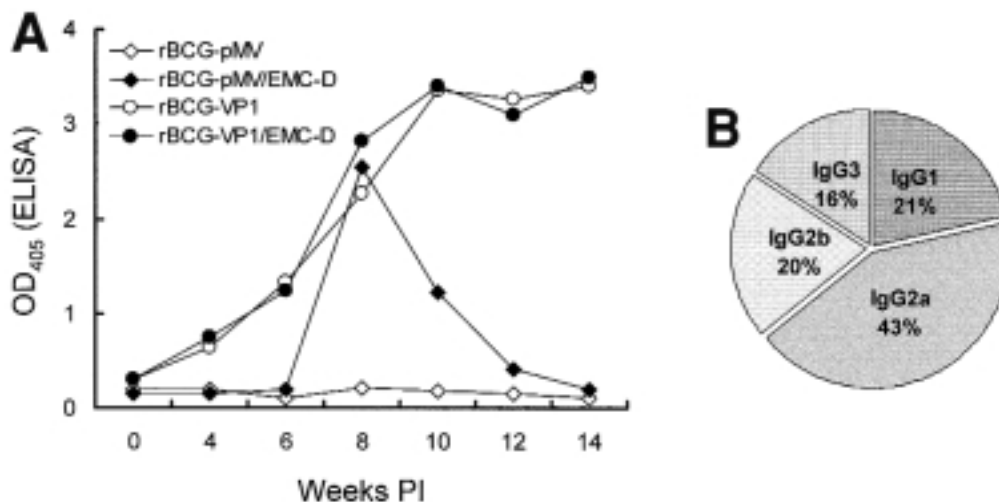


FIG. 4. Kinetics (A) and isotyping (B) of VP1-specific IgG in the immunized mice. The 6-week-old SJL/J male mice ($n = 20$ /group) were inoculated with a single dose of 10^7 cfu i.p. of control rBCG-pMV and rBCG-VP1, respectively. Half of each group ($n = 10$) was challenged with the EMC-D virus at week 6 after immunization. A: Sera obtained biweekly from the immunized mice were analyzed for the titer of VP1-specific IgG by ELISA (A_{405}). Data are mean absorbency values for each group ($n = 10$, $P < 0.01$) at the indicated time points. B: Isotype analysis of the anti-VP1 IgG from rBCG-VP1-immunized mice at week 10. Data are cumulative percentages for each IgG subclass. OD, optical density; PI, postinfection.

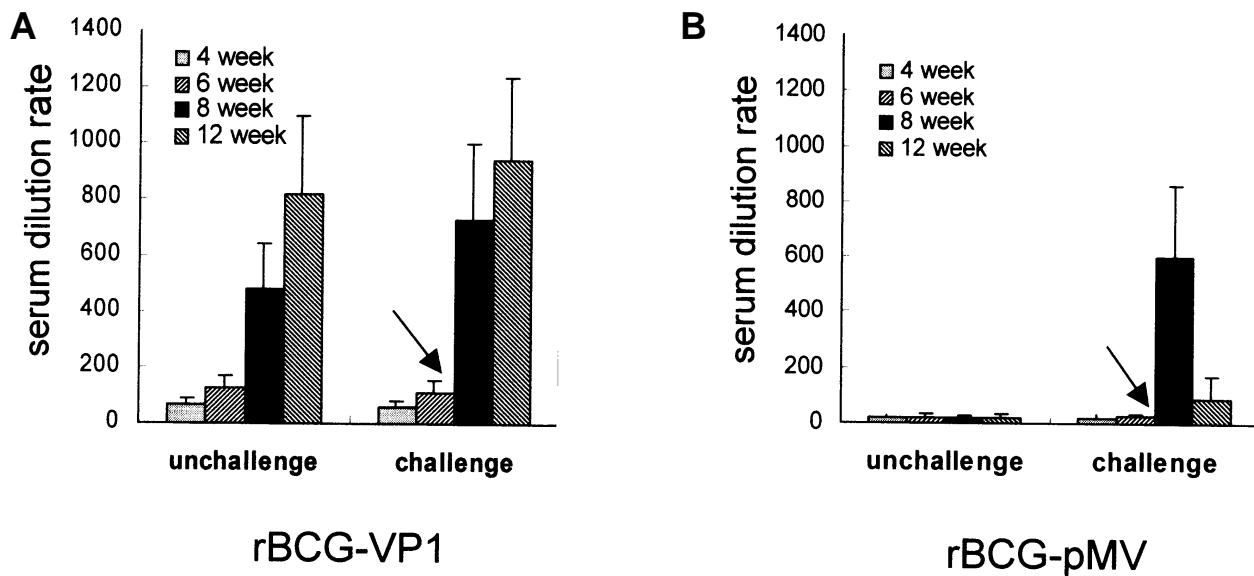


FIG. 5. Titer of the neutralizing antibody in the sera obtained from the mice immunized with rBCG-VP1 ($n = 10$) (A) and control rBCG-pMV ($n = 10$) (B). Twofold serial dilutes of serum starting at 1:10 were mixed and incubated with 125 TCID₅₀ U of the EMC-D virus for 1 h. HeLa cell suspension was added to each well and then incubated for an additional 7 days. Cells were stained with 1% crystal violet. Titers are the mean values of maximum dilution rates of serum preventing 50% cytopathology. Data are geometric means \pm SD of the serum dilution rates of each group ($n = 8-10$, $P < 0.05$) at the time points shown. The arrow indicates the time point of challenge with the highly diabetogenic EMC-D virus.

of anti-VP1 antibody ranged from 260 to 6,500 when expressed by serum dilution rate. VP1-specific IgG titer reached the maximum 10 weeks after the primary inoculation, and the titer was maintained throughout the next 4 weeks. Control mice immunized with rBCG-pMV revealed no VP1-specific antibody in 1/10 diluted serum. The isotype analysis of the IgG subclasses indicated a prevalence of IgG2a in the vaccinated mice, whereas other isotypes were about half of the IgG2a in concentration at week 10 after immunization (Fig. 4B).

Sera taken at 4, 6, 8, and 12 weeks after immunization were tested for their neutralizing capacity against the EMC-D virus. The titer of neutralizing antibody in the rBCG-VP1-immunized mice increased over time ($P < 0.05$). It ranged from 40 to 10³ at 125 TCID₅₀ U throughout the experimental period (Fig. 5A). Neutralizing activity was not detected in sera obtained from the control mice immunized with rBCG-pMV (Fig. 5B). When the rBCG-VP1-immunized mice were challenged with the EMC-D virus at week 6, VP1-specific antibodies and neutralizing capacity were increased >30% at week 8 compared with those of the unchallenged mice (Figs. 4 and 5A). In the same challenge, however, control BCG-immunized mice also showed a marked enhancement in VP1-specific neutralizing antibodies for a short period that then waned rapidly (Figs. 4 and 5B).

VP1-specific DTH responses. The DTH to the VP1 of the EMC-D virus was studied in the immunized mice 6, 8, and 12 weeks after the primary inoculation. At each time point, rBCG-VP1-immunized mice developed remarkable VP1-specific DTH responses ($n = 6$, $P < 0.01$) compared with control mice inoculated with rBCG-pMV or PBS only (Fig. 6). As shown in Fig. 6, DTH response in rBCG-VP1-immunized mice gradually increased as time passed throughout the entire observation period, although it slightly waned in control BCG-immunized mice during the same period. The DTH responses shown in mice were similarly repeated in guinea

pigs. Guinea pigs immunized with rBCG-VP1 as described in RESEARCH DESIGN AND METHODS showed strong and clear DTH responses to VP1 in the skin test ($P < 0.001$) when challenged with purified VP1 protein (data not shown). In both cases, control BCG-inoculated animals showed more or less than half the intensity of DTH responses to VP1 versus the rBCG-VP1-immunized group.

VP1-specific lymphocyte proliferation and long-lasting protective immunity. At 10 months after immunization, mice were still resistant to the EMC-D-induced diabetes. To identify whether the rBCG-VP1-mediated protective immu-

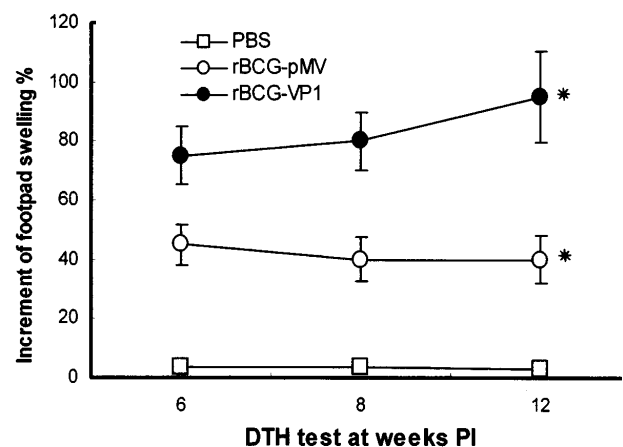


FIG. 6. DTH responses to rVP1 in mice immunized with rBCG-VP1. The 6 SJL/J mice in each group were immunized intraperitoneally with the indicated rBCG. Six, 8, and 12 weeks postinfection (PI), DTH responses were determined by footpad swelling assay as described in RESEARCH DESIGN AND METHODS. Data are means \pm SD (error bars) increases in footpad thickness. * $P < 0.01$ vs. results for control mice.

TABLE 2
Enumeration of VP1-specific antibody-producing cells by ELISPOT in the spleens of the rBCG-VP1-immunized mice

Used for immunization	Spot-forming cells/10 ⁶ splenocytes (n)			
	10 weeks		10 months	
	rVP1 (2 µg/ml)	PPD (5 µg/ml)	rVP1 (2 µg/ml)	PPD (5 µg/ml)
PBS only	10 ± 3	13 ± 3	11 ± 4	12 ± 5
rBCG-pMV	21 ± 5	370 ± 48	15 ± 6	60 ± 4
rBCG-VP1	138 ± 21	420 ± 75	18 ± 5	75 ± 4

Data are means ± SD. Splenocytes taken from 3 immunized mice 10 weeks and 10 months, respectively, after inoculation were stimulated with affinity-purified rVP1 protein and PPD for 24 h. Antibody-producing cells were then counted.

nity is associated with cellular immunity, spleens were examined for VP1-specific lymphocytes in immunized mice 10 weeks and 10 months after immunization. At 10 weeks after immunization, more than 100 of the VP1-specific antibody-producing B-cells were detected per 10⁶ splenocytes in spleens of the rBCG-VP1-immunized mice in the ELISPOT test (Table 2). At 10 months, however, VP1-specific antibody-producing B-cells were barely detected in spleens (Table 2), and VP1-specific IgG was almost undetectable in the sera of rBCG-VP1-immunized mice (data not shown) when examined without further boosting or challenge.

A proliferative response to VP1 was evaluated with mouse splenic T-cells in the presence of rVP1. Considerable amounts of VP1-specific T-cells were detected at week 10 after immunization (Fig. 7A), and the proliferative response was markedly enhanced at 10 months in the immunized mice (Fig. 7B). [³H]thymidine absorbed by T-cell-enriched splenocytes at week 10 after immunization in the absence and presence of 5 µg/ml rVP1 antigen ranged from 4,800 to 6,200 cpm (5,460 ± 620 cpm) and from 33,500 to 44,800 cpm (39,300 ± 1,600 cpm), respectively. On the other hand, at 10 months, [³H]thymidine ranged from 2,200 to 3,500 cpm (2,800 ± 530 cpm) and from 77,000 to 99,700 cpm (88,800 ± 9,270 cpm), respectively, in the same conditions as above. Differences in the scale of the SI between 10 weeks and 10 months in Fig. 7 are discussed below.

DISCUSSION

EMC-D virus-induced diabetes in SJL/J male mice is a well-defined animal model for human type 1 diabetes. The EMC virus, which belongs to the *Picornaviridae*, is a (+)-sense single-stranded RNA virus. As with other picornaviruses, it consists of 4 capsid proteins, 3 of which (VP1, VP2, and VP3) are exposed on the surface of the virion. Among the 3, VP1, the major outer capsid protein, is believed to play an important role for virus attachment and immunogenicity (31,32).

Our work was initiated to apply the BCG vector system for the prevention of EMC-D virus-induced diabetes. We have constructed rBCG (rBCG-VP1) by introducing the VP1 cDNA of the EMC-D virus. The rBCG-VP1 was tested for its immunogenic capacity against the EMC-D virus in SJL/J mice.

The VP1 expression from the rBCG lasted longer than at least 4 weeks after a single heat shock in vitro (Fig. 2A). The rVP1 expressed in rBCG could be detected with the antiserum obtained from EMC-D-infected SJL/J mice (Fig. 2A), which indicates that the rVP1 retains antigenicity, at least in part, similar to that of the authentic VP1 protein of the EMC virus. The rBCGs recovered from the immunized mice 8 weeks after immunization still had the plasmid containing the VP1-cDNA (Fig. 2B), which kept the capacity to express rVP1 (Fig. 2C), suggesting that the rBCG is also genetically stable in vivo.

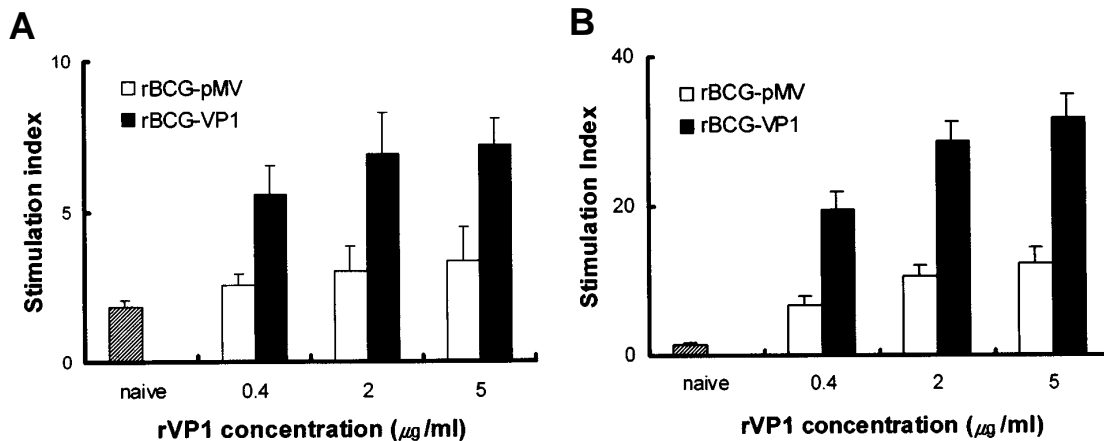


FIG. 7. Lymphoproliferative responses to rVP1 antigen stimulation in T-cell-enriched splenocytes of immunized mice. Spleens were removed from mice ($n = 3$ /group) at 10 weeks (A) and 10 months (B) after immunization with rBCG-VP1 and control BCG (rBCG-pMV), respectively. Lymphocyte proliferation and labeling were performed as detailed in RESEARCH DESIGN AND METHODS and are expressed as means ± SD of SIs from the assays, each in triplicate determinations ($P < 0.01$). Naive indicates a lymphoproliferative response of unimmunized normal mice to the rVP1 antigen stimulation at a concentration of 0.4 µg/ml.

None of the 10 SJL/J male mice immunized with rBCG-VP1 became diabetic when challenged at week 6 with the highly diabetogenic EMC-D virus (Fig. 3, Table 1). Our additional studies showed that these protections resulted from the effective humoral and cellular immunity against the EMC-D virus induced by rBCG-VP1 in the immunized mice. Actually, rBCG-VP1 produced very small amounts of VP1 protein (Fig. 2C) compared with pRSET-VP1-transformed *E. coli* (Fig. 2C) but was efficient in inducing an immune response against EMC-D infection in vivo as described previously (17). That may be not only because of the adjuvant potential of BCG (14) but also because of the BCG-mediated cellular immunity generated by continuous expression of antigen molecules released from the rBCG, which replicate actively in the phagocytic macrophages (15,16) for a long period of time in vivo.

When rBCG-VP1-immunized mice were challenged at week 4 after immunization, ~50% of the mice showed diabetes symptoms (glycosuria) within a week. Most of them, however, had recovered during the following weeks when tested for serum glucose levels (data not shown). This finding indicates that a single-dose inoculation of rBCG-VP1 requires at least 6 weeks to induce appropriate immune responses against 10^6 pfu of the EMC-D virus.

On the contrary, 8 out of 10 mice inoculated with control BCG (rBCG-PMV, BCG transformed with vector only) developed diabetes a week or so after challenge at week 6 with the EMC-D virus (Fig. 3, Table 1). When considering the fact that the incidence of diabetes from the EMC-D virus is >90% when inoculated into susceptible mice, the prevention of EMC-D-mediated diabetes in the rBCG-VP1-immunized mice is thought to be because of the induction of specific immunity against the EMC-D virus. Control BCG-inoculated mice developed diabetes 1 to 2 days later than the normal mice when challenged with the EMC-D virus, which suggests that the BCG-mediated nonspecific immune enhancement may impede the viral infection.

Marked enhancement of VP1-specific IgG (Fig. 4A) and neutralizing antibody (Fig. 5A) at week 8 after primary immunization seems to be because of the viral challenge at week 6 that resulted in boosting effects on the rBCG-VP1-immunized mice. The effect decreased to the level of unchallenged mice at week 12. The same enhancement of the VP1-specific IgG and neutralizing antibody appeared in the control BCG-immunized mice at 2 weeks after challenge (Figs. 4A and 5B). This enhancement, however, is thought to be because of the infection and replication of the EMC-D virus rather than the boosting effects. These enhancements rapidly decreased during the following weeks.

Because mycobacteria are rapidly phagocytosed and are located within the episomal compartments of phagocytic cells, we expected that rBCG would be also effective to induce T-cell-mediated antibody production. The prevalence of IgG2a subclasses in the VP1-specific antibodies in the rBCG-immunized mice (Fig. 4B) suggests that the immune response induced by rBCG-VP1 is associated with Th1 cell-mediated cellular immunity.

We did not investigate the cytotoxic T-lymphocyte (CTL) response in the immunized SJL/J mice because we could not prepare appropriate target cells. However, several reports have already shown that rBCG induces effective CTL (23,33,34) by unknown mechanisms. If dendritic cells are involved in the BCG-mediated preventive immune response

as suggested previously (35,36), then even small amounts but continuous expression of rVP1 in rBCG would be enough to induce an effective CTL response against the EMC-D virus by the cross-priming capacity of dendritic cells (37). Instead of CTL assay, we have investigated the VP1-specific T-cells by examining DTH response and T-cell proliferation. As shown in Figs. 6 and 7A, the rBCG-VP1 was very effective in inducing T-cell-mediated immunity in the rBCG-VP1-immunized animals. Particularly, VP1-specific DTH response was confirmed in both rBCG-VP1-immunized mice and guinea pigs. rVP1 itself was able to prevent the EMC virus-induced diabetes in mice (26). However, the subunit vaccine is well known to have its own limitation in inducing long-lasting cellular immunity. Within a few weeks, a single-dose vaccination with rBCG-VP1 was not as effective as subunit VP1 vaccination in inducing neutralizing antibody (data not shown). But during the following periods up to 10 weeks, rBCG-VP1-mediated protective immune responses were markedly enhanced and then maintained for several weeks (Fig. 4A). During this period, rBCG-VP1-mediated immune responses against the EMC-D virus were much more potent than those induced by a subunit vaccine even in humoral immunities.

In addition, rBCG was very effective in inducing long-lasting VP1-specific T-cell immunity as shown in Fig. 7B. After 10 months, rBCG-VP1-immunized mice had few VP1-specific antibody-producing B-cells in the spleen (Table 2) and an undetectable level of VP1-specific antibodies in the blood stream (data not shown). Nevertheless, all of the rBCG-VP1-immunized mice ($n = 3$) were still resistant to the virus-induced diabetes when challenged with the EMC-D virus. Particularly noteworthy is the fact that VP1-specific splenic T-cell proliferation was markedly enhanced (2 to 3 times) at 10 months compared with that at 10 weeks after a single immunization (Fig. 7). In the proliferation assay, the average cpm at 10 months ($88,800 \pm 9,270$ cpm) was 2.25 times as high as that at 10 weeks ($39,300 \pm 1,600$ cpm) but was shown to be 4 to 5 times as high in Fig. 7B when expressed by the SI. The reason is that the control cpm (in the absence of antigen) at 10 months ($2,800 \pm 530$ cpm) was half of the value at 10 weeks ($5,460 \pm 620$ cpm). These results suggest that the rBCG-mediated VP1-specific cellular immunity at 10 months is associated with VP1-specific memory T-cells rather than active T-cells. Our results, however, do not exclude the possibility that humoral immunity was also involved in the long-term preventive immunity in our rBCG vaccination because of many memory B-cells remaining in bone marrow, which is the reason that the VP1-specific memory B-cells could not be detected in our spleen ELISPOT tests but could possibly provide protective humoral immunity.

Coxsackievirus, which belongs to *Picornaviridae*, has been thought to induce human autoimmune type 1 diabetes by either molecular mimicry or bystander T-cell activation (38). For a long time, attention has focused on the molecular mimicry between the 2P-C of Coxsackievirus B4 and the islet autoantigen GAD (39–41). However, recent emerging evidence from transgenic mice experiments strongly suggests that bystander T-cell activation rather than molecular mimicry causes the Coxsackievirus-induced type 1 diabetes in mice (42–44). Thus, vaccination against a viral infection in the pancreas may be effective for preventing

Coxsackievirus-mediated autoimmune type 1 diabetes, and the rBCG system demonstrated in this article may be applicable for developing a preventive vaccine against Coxsackievirus-induced diabetes.

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