

Establishment of a Foreign Antigen Secretion System in Mycobacteria

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In order to develop recombinant *Mycobacterium bovis* BCG into a useful multivaccine vehicle, we established a foreign antigen secretion system in mycobacteria in which an extracellular α antigen of *Mycobacterium kansasii* was utilized as a carrier. By using this system, a B-cell epitope (Glu-12-Leu-Asp-Arg-Trp-Glu-Lys-Ile-19) of human immunodeficiency virus type 1 p17^{gag}, which was identified by a fusion protein-based method, has been successfully obtained from BCG along with the α antigen. This is the first report of expression and secretion of a foreign viral antigen from BCG. It is possible that the system can become a universal vaccination vehicle applicable to protection against various infectious diseases.

The most widely used vaccine against tuberculosis is the live vaccine *Mycobacterium bovis* BCG. This vaccine has superb adjuvant activity, being able to induce both the humoral and cell-mediated immune systems with a low rate of serious complications. A host-vector system of BCG, which is a fundamental aspect of the development of recombinant live BCG vaccines, has recently received increased attention (2, 7). Recent work on successful transformation in mycobacteria by Snapper et al. (26) enabled us to express a foreign gene in BCG.

In general, it is accepted that an effective subunit vaccine development strategy must take into account factors such as humoral and cellular immune responses, adjuvants, and a carrier. In addition, it is important for a recombinant BCG vaccine to have foreign antigens continuously secreted from the live BCG. These antigens must then be processed and presented dependably on the cell surfaces of macrophages to continuously stimulate a host immune system. Such a repetitive stimulation at the B- and T-cell level would result in a long-lasting immune response. Furthermore, the system involving secretion would have some advantages for the excretion of a large amount of recombinant antigen favorable to better stimulation of the host immune system and for the release of a foreign protein which may be toxic for or degraded in BCG. In this respect, it is of particular interest to develop a novel approach to a foreign antigen secretion system of BCG. Thus, we have cloned and analyzed several genes for proteins secreted by mycobacteria, such as the α antigen (α -B) (15), MPB64 (31), and MPB70 (29) derived from BCG and the α antigen from *Mycobacterium kansasii* (α -K) (14).

In this paper we describe the establishment of a foreign antigen secretion system in mycobacteria. We also describe successful secretion of a B-cell epitope of human immuno-

deficiency virus type 1 (HIV-1) (1, 20, 21) p17^{gag} from BCG by using an α -K secretion vector.

MATERIALS AND METHODS

Reagents. All enzymes used, a DNA ligation kit, and a *KpnI* linker were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Peroxidase-conjugated goat anti-rabbit immunoglobulin G and peroxidase-conjugated goat anti-mouse antibody were from Dako Co., Ltd. (Copenhagen, Denmark) and Bio-Rad Laboratories (Richmond, Calif.), respectively. An anti-HIV-1 p17^{gag} mouse monoclonal antibody (MAb) was purchased from Chemicon International, Inc.

Bacterial strains and cultures. The mycobacterial strains used were *Mycobacterium smegmatis* Rabinowitchi (R) and the BCG Tokyo vaccine strain. Cultures of these strains were grown in Middlebrook 7H9 broth (Difco Laboratories) containing albumin-dextrose complex (Difco) and 0.05% Tween 80 or in Souton medium (27) at 37°C. *Escherichia coli* HB101 competent cells were purchased from Takara Shuzo. *E. coli* EQ192 was obtained from H. Kagawa (8). These *E. coli* strains were grown in YT broth (31).

Construction of α -K secretion vectors. The *E. coli*-mycobacteria shuttle plasmid pIJ666-pAL5000 library was obtained from T. Kieser (26). Two isolates (pIS18 and pIS27) from this library were digested with *KpnI* and dephosphorylated with bacterial alkaline phosphatase. Additionally, plasmid pKAH20 (14) was digested with *HincII*, and the resulting 2.0-kbp fragment was isolated by the DE81 paper method (15). This fragment was ligated with *KpnI* linkers by using a DNA ligation kit. After phenolization and ethanol precipitation, the DNA fragment was digested with *KpnI* to convert it to a *KpnI* fragment. This was then ligated with the *KpnI*-digested pIS18 and pIS27 (see Fig. 2) and transformed into *E. coli* HB101. The resulting plasmids were named pIJK-1 and pIJK-2, respectively.

Construction of an HIV-1 p17^{gag} B-cell epitope secretion vector. Epitope mapping of HIV-1 p17^{gag} was performed by the fusion protein-based method (8, 30). We prepared chemically synthesized DNA fragments encoding various regions of p17 and introduced them into the *BamHI*-*PstI* sites of

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pUR292 (24), which was capable of expressing the β -galactosidase fusion proteins in *E. coli* EQ192. The β -galactosidase-p17 peptide fusion proteins were analyzed by Western immunoblot assay using the anti-p17^{gag} MAb (data not shown). The MAb recognized eight amino acids (Glu-12-Leu-Asp-Arg-Trp-Glu-Lys-Ile-19) in the p17 antigen. This sequence was identical to the epitope mapped with synthetic peptides by Papsidero et al. (19). On the other hand, the α -K gene had a unique *Pst*I site in its 3'-terminal region (14). We used this site for introducing the epitope-encoding DNA fragment (Fig. 6). Two oligonucleotides, whose sequences were 5'-GGGCGAGCTGGACCGTGGGAGAAGATCGC CAGCCTGGGCGCCCCGCTGA-3' (for the upper strand) and 5'-AGCTTCAGCGGGCGCCAGGCTGGCGATCTTCTCC CACCGTCCAGCTCGCCCTGCA-3' (for the lower strand), were synthesized by a 380A automated DNA synthesizer (Applied Biosystems Inc.) and purified as described previously (15). These oligomers were annealed to generate a *Pst*I-*Hind*III fragment possessing sequences for the p17 epitope and for the COOH-terminal six amino acids of α -K. The fragment was ligated with *Pst*I-*Hind*III double-digested pKAH20, giving rise to pKAH21. This plasmid possessed the gene for the fusion protein in which the sequence for nine amino acids (Gly-Glu-Leu-Asp-Arg-Trp-Glu-Lys-Ile) was inserted between Gln-279 and Ala-280 of α -K. To isolate a DNA fragment containing the fusion protein gene, we inserted a synthetic *Kpn*I linker into the *Hind*III site of pKAH21. The resulting plasmid was digested with *Kpn*I, generating a 1.4-kbp *Kpn*I fragment which was subcloned into the *Kpn*I site of pIS18. The final construct was named pIJKHIV-1. The gene manipulations mentioned above were all performed in *E. coli* HB101.

Immunological techniques. Transformation of mycobacteria with the secretion vectors pIJK-1, pIJK-2, and pIJKHIV-1 was performed by electroporation by the method described by Snapper et al. (26). The transformants were grown in 200 ml of Souton medium for 30 to 40 days. After the cells were harvested, the culture supernatant was filtered through a Millipore filter (pore size, 0.45 μ m) and was 80% saturated with ammonium sulfate at 4°C for 24 h. The precipitated proteins were dissolved in 2 ml of 25 mM Tris hydrochloride buffer (pH 7.5) and dialyzed against the same buffer. A small portion (10 μ l) of the protein preparation was electrophoresed on a sodium dodecyl sulfate-15% polyacrylamide Laemmli gel (11) and electroblotted to a nitrocellulose membrane filter. In the case of α -K secretion, immunodetection of the antibody-reactive protein was performed as described previously (15) by using absorbed anti- α -K serum prepared by the method of Tasaka and Matsuo (28). Rabbits were intramuscularly inoculated with α -K antigen combined with Freund incomplete adjuvant (Iatron, Tokyo, Japan). Partially purified IgG antibody (40 mg/ml) was mixed with the same volume of 20-mg/ml partially purified α antigen of *Mycobacterium tuberculosis* (α -T), incubated at 37°C for 1 h and at 4°C overnight, and centrifuged at 2,000 \times g for 30 min. The supernatant was referred to as the absorbed anti- α -K serum. Additionally, in the immunodetection of the epitope- α -K fusion protein, we used the anti-p17^{gag} MAb as a primary antibody and the peroxidase-conjugated goat anti-mouse antibody as a secondary antibody.

Purification of α -K secreted from recombinant clones. The protein solution prepared from the culture filtrate of BCG184 (harboring pIJK-1) or *M. smegmatis* 274 (harboring pIJK-2) was desalted with a PD-10 column (Pharmacia) equilibrated with distilled water and loaded onto an FPLC Superose 12 column (10 mm [diameter] by 300 mm; Pharmacia) which

had previously been equilibrated with 50 mM Tris hydrochloride (pH 7.5)-0.15 M NaCl buffer. The fraction corresponding to that of α -K, which was eluted 40 min after injection with a flow rate of 0.4 ml/min, was collected and desalted with a PD-10 column. The protein fraction was concentrated and separated by reverse-phase high-performance liquid chromatography (HPLC) (TSKgel Phenyl-5PW RP column; 4.6 mm [diameter] by 75 mm; Tosoh Co. Ltd., Tokyo, Japan) with a linear gradient of acetonitrile (20 to 50% for 25 min) in 0.05% trifluoroacetic acid. A portion (400 μ l) of each fraction was supplemented with 5 μ g of carrier bovine serum albumin, and the protein mixture was precipitated in 5% trichloroacetic acid. Western blot analysis for the protein was carried out as described above. The fraction including antibody-reactive protein was analyzed by a 470A gas phase protein sequencer (Applied Biosystems).

RESULTS

Isolation of a single plasmid replicable in mycobacteria from the pIJ666-pAL5000 library. To facilitate the construction of α -K secretion vectors, it was initially necessary to purify a single plasmid from the mixed shuttle plasmid pIJ666-pAL5000 library (26), in which *Bam*HI-digested pIJ666 (9) was randomly inserted at a variety of *Mbo*I sites in pAL5000 (10). The entire DNA sequence of the plasmid pAL5000, the first reported mycobacterial plasmid, has been determined (22). It has five open reading frames, and it has been shown that a foreign gene can be inserted into a unique *Kpn*I site located within the ORF3 open reading frame without preventing its replication (22). It was therefore presumed that the shuttle plasmid, into which pAL5000 linearized within ORF3 by *Mbo*I partial digestion, was cloned between two *Bam*HI sites of pIJ666 and possibly would replicate in mycobacteria. An attempt was made to isolate this plasmid. There were two *Hind*III sites 342 bp internal to both ends of the *Bam*HI-digested pIJ666 large fragment (9). Accordingly, the location of *Mbo*I sites used for cloning could be determined by estimating the size of the DNA fragment derived from a *Kpn*I-*Hind*III double-digested shuttle plasmid. *E. coli* HB101 was then transformed with the library, and 36 clones of chloramphenicol-resistant transformants were isolated. These plasmids were double digested with *Hind*III and *Kpn*I and electrophoresed on a 4% polyacrylamide gel (Fig. 1). In this way, pIS18 and pIS27 were obtained as potential candidates and were then used to construct the α -K secretion vectors.

Secretion of α -K from BCG and *M. smegmatis*. The strategy for construction of α -K secretion vectors is shown in Fig. 2. The constructed vectors (pIJK-1 from pIS18 and pIJK-2 from pIS27) consist of a promoter, a signal peptide region, and a structural gene for α -K. They were introduced into BCG Tokyo and *M. smegmatis* R by electroporation. These experiments yielded at least 100 to 500 kanamycin-resistant transformants per μ g for BCG and 10 kanamycin-resistant transformants per μ g for *M. smegmatis*.

We analyzed the filtrate from the culture of the transformants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a Western blot assay. The results are presented in Fig. 3. In BCG184 harboring pIJK-1, as well as BCG274 and *M. smegmatis* 274 harboring pIJK-2, the absorbed anti- α -K serum detected a single reactive protein which migrated with a molecular weight similar to that of the purified native α -K (Fig. 3, lanes 9, 10, and 12).

Purification of antibody-reactive proteins. For further confirmation, we purified the antibody-reactive proteins from

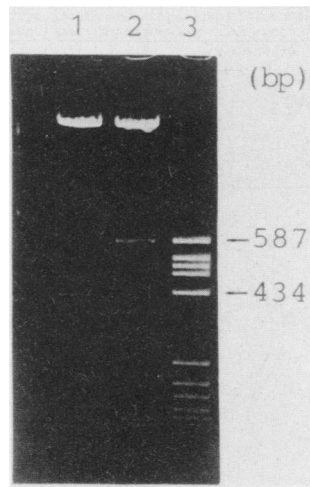


FIG. 1. Single-plasmid isolation from pIJ666-pAL5000 library. *Hind*III-*Kpn*I-digested pIS18 (lane 1) and pIS27 (lane 2) were electrophoresed on an 8% polyacrylamide gel with *Hae*III-digested pBR322 fragment (lane 3) as size markers.

the culture media of BCG184 and *M. smegmatis* 274 by a two-step process involving HPLC and determining the NH₂-terminal amino acid sequences. Figure 4 shows the chromatograms obtained by using a TSKgel Phenyl-5PW RP column in the second step. In both instances, the antibody-reactive proteins of BCG184 and *M. smegmatis* 274 were eluted as a primary peak in this situation (Fig. 4a, lane 2, and Fig. 4b, lane 3). The purified protein was then analyzed with a protein sequencer. The NH₂-terminal sequences of the

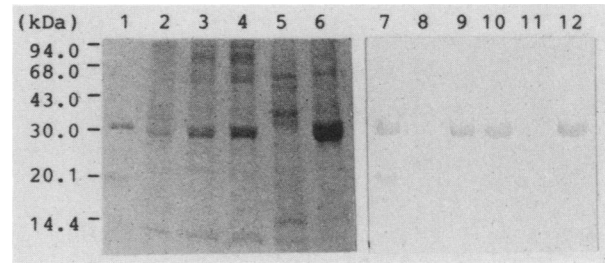


FIG. 3. Analysis of the culture filtrate of recombinant mycobacterial clones. Lanes 1 to 6 were stained with Coomassie brilliant blue to visualize all proteins. Lanes 7 to 12, protein which reacted with absorbed anti- α -K serum (see Materials and Methods). Lanes 1 and 7, 2 μ g of purified α -K antigen; lanes 2 and 8, control culture filtrate of BCG harboring pIS18; lanes 3 and 9, culture filtrate of BCG184; lanes 4 and 10, culture filtrate of BCG274; lanes 5 and 11, control culture filtrate of *M. smegmatis* R; lanes 6 and 12, culture filtrate of *M. smegmatis* 274. Molecular size standards (Pharmacia) are as follows: α -lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa), and phosphorylase B (94.0 kDa).

proteins purified from BCG184 and *M. smegmatis* 274 are presented in Fig. 5. They were completely identical with that of α -K (14) but not with the sequence from α -B (15), and no unprocessed protein with the signal peptide was found, suggesting that the mature α -K protein was secreted from these mycobacterial species.

Secretion of a B-cell epitope of HIV-1 p17^{gag} from BCG. To prove the applicability of the α -K secretion vectors, we attempted to induce BCG to secrete an α -K chimera protein fused with one of the B-cell epitopes of HIV-1 p17^{gag} from BCG. In the development of a vaccine against acquired immune deficiency syndrome (AIDS) caused by HIV-1, there is a growing interest in the *gag* and *pol* antigens, which are believed to be more rigidly conserved than the antigen of the *env* gene. For a model for a subunit AIDS vaccine, we focused on p17^{gag} B-cell epitopes which could easily be mapped with MAbs by a fusion protein-based or synthetic peptide-based method. Because one MAb was found to recognize eight amino acids (Glu-12 through Ile-19) in p17, synthetic oligonucleotides coding for this peptide were inserted into the 3'-terminal region of the α -K gene (Fig. 6). The culture medium from BCG transformed with the vector pIJKHIV-1, named BCG18HIV, was analyzed by Western blot assay using the MAb against p17 (Fig. 7). Although no protein was detected in the control culture medium (BCG184; Fig. 7, lane 7), a reactive protein with a slightly larger molecular weight than that of α -K (lane 1) was detected in the BCG18HIV culture medium (lane 8). This indicated that the B-cell epitope along with the α -K carrier was secreted from the BCG cells.

DISCUSSION

In this paper, we report the establishment of a foreign antigen secretion system in mycobacteria. By using the secretion apparatus for α -K, the B-cell epitope of HIV-1 *gag* antigen was effectively excreted from BCG, suggesting that the α -K secretion vector could be widely used to excrete other foreign antigens. The reason why we used the cross-reactive antigen α -K was that it is the most suitable carrier for the secretion of foreign antigens because of its structural similarity to α -B. More importantly, α -K, which possesses the species-specific antigenic determinant (28), exhibits sero-

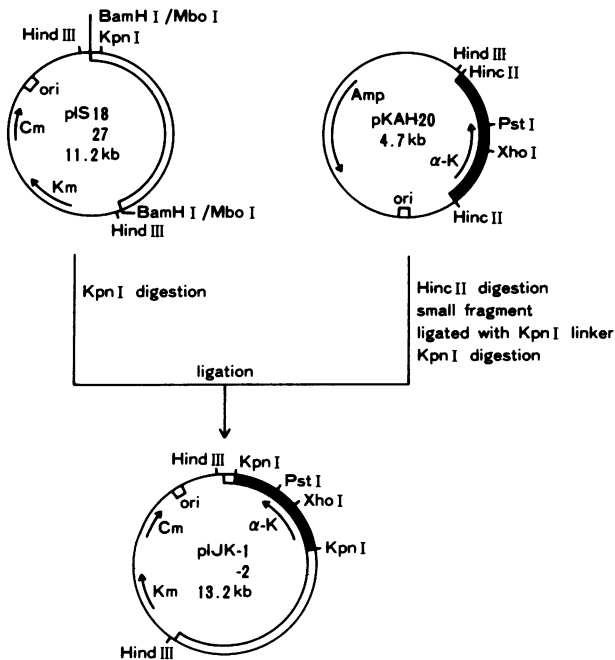


FIG. 2. Schematic outline of the construction of an α -K secretion vector. The mycobacterial DNA is represented by thick lines. The thick black lines show a DNA fragment containing the α -K gene. Amp, Cm, and Km indicate ampicillin, chloramphenicol, and kanamycin resistance genes, respectively.

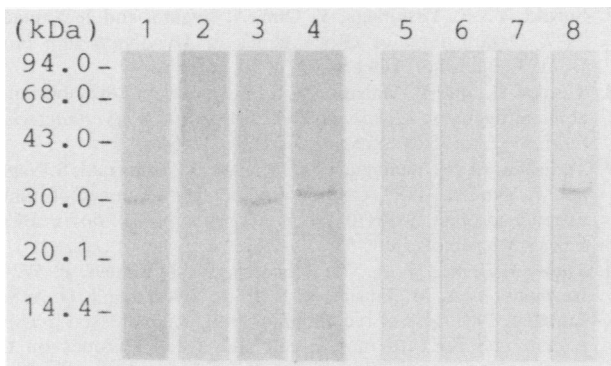


FIG. 7. Western blot analysis of culture filtrate of recombinant BCG clones. Lanes 1 and 5, 2 μ g of purified α -K antigen; lanes 2 and 6, control culture filtrate of BCG harboring pIS18; lanes 3 and 7, culture filtrate of BCG184; lanes 4 and 8, culture filtrate of BCG18HIV. Lanes 1 to 4 show proteins reactive to the absorbed anti- α -K serum. Lanes 5 to 8 show proteins reactive to the anti-p17^{gag} MAbs. Molecular size standards are as described in the legend to Fig. 3.

people with extremely low risk (12), it can be given at birth, and it is available at low cost. It could, therefore, offer the possibility of creating a kind of polyvalent vaccine to protect people against a wide variety of bacterial, viral, and parasitic agents with only one injection at birth.

Immunity to facultative intracellular bacteria, including BCG, is complex. There are crucial events involving the interaction between macrophages and several T-cell subsets, including helper, delayed-type hypersensitivity-inducing, and cytotoxic T cells. It is particularly important to determine which subset is stimulated by a recombinant BCG expressing foreign T-cell epitopes, either intracellularly or extracellularly. It is generally accepted that to develop cytotoxic T cells, which are involved in protection from viral and parasitic infections (23), antigens must be processed intracytoplasmically and presented with class I major histocompatibility complex molecules (16). Although little is currently known about the molecular behavior of phagocytosed BCG cells within macrophages, secreted proteins might be expected to leak from phagosomes and enter the endogenous processing pathway, as was reported by Romero et al. (23), who studied the circumsporozoite protein of malaria parasites. Moreover, if BCG cells could escape from phagosomes into the cytoplasm, it might be also expected that a recombinant antigen secreted extracellularly from BCG could be processed through the endogenous pathway to stimulate cytotoxic T cells. Studies of these problems should contribute to a better understanding of antigen processing and presentation with class I or class II major histocompatibility complex molecules, which is one of the main fields of interest for many immunologists and molecular biologists (3). Elucidation of these mechanisms may lead to the design and development of ideal recombinant BCG vaccines.

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