Construction of Recombinant Infectious Laryngotracheitis Virus Expressing the LacZ Gene of E. coli with Thymidine Kinase Gene

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ABSTRACT. We constructed the recombinant infectious laryngotracheitis virus (ILTv), CE strain, containing the LacZ gene of E. coli in the thymidine kinase gene. The growth property of the recombinant virus was almost the same as parental CE strain in chicken embryo fibroblasts. The recombinant CE strain of ILTV could be used as a live vaccine vector.—KEY WORDS: ILTV-CE strain, recombinant ILTV, TK gene.

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens caused by the ILT virus (ILTv). The disease is important to the intensive poultry industry, because outbreaks of ILT have resulted in high mortality among chickens and reduction in egg production [2].

ILTv is classified as one of the alphaherpesvirinae and is known as Gallid herpesvirus 1 [10]. The genome of ILTV is linear double stranded DNA approximately 155 kilobase pairs (kbp) in length [19]. The genome structure is composed of a long unique region and a short unique region flanked by the inverted repeat sequence, which is similar to that of pseudorabies virus (PRV), equine herpesvirus 1 (EHV-1) and infectious bovine rhinotracheitis virus (IBRV) [15]. In recent years, some herpesviruses, herpesviruses of turkeys (HVT) [20, 23], IBRV [1, 13], PRV [26], herpes simplex virus (HSV) [17] and varicella-zoster virus [16] have been used as live virus vectors. In many cases, thymidine kinase (TK) gene was used as a selectable marker gene for homologous recombination.

The live vaccines have been used for the prevention of ILT, and various methods of vaccination have been tried. However, no successful vaccination by the subcutaneous (s.c.) or intramuscular (i.m.) route has been reported [8]. Moreover, vaccination of chickens under 14-day-old has failed to confer sufficient immunity [3]. Marked immunosuppression in ILT immunization was also observed when chickens were given a live Newcastle disease (ND) vaccine either simultaneously or shortly after vaccination with live ILTV vaccine [9].

In previous reports [24, 25], we referred to cell-associated (C-A) ILT vaccine, using the CE strain [14]. The CE strain, a C-A vaccine strain, was developed by The Chemo-Sero Therapeutic Research Institute (Kumamoto, Japan). This vaccine eliminated the several defects described above, as follows: (a) The s.c. or i.m. administration of the C-A vaccine gave good protection against challenge exposure [25]. (b) More than 60% of day-old chickens vaccinated with the C-A vaccine maintained immunity for 10 weeks post-vaccination (PV) [25]. (c) The development of immunity was not hindered by further vaccination with Newcastle disease (ND) and infectious bronchitis (IB) combined (ND-IB) live vaccine [25]. Moreover, the CE strain can grow in chicken embryo fibroblasts (CEF). We therefore tried to make recombinant ILTV with the CE strain.

In this report, we describe the procedures for construction of recombinant ILTV with the TK gene, and discuss the possibility of using ILTV as a new vector for poultry live vaccine.

CEF monolayer was prepared on a 10 cm Petri dish and inoculated with CE strain. When the cytopathic effect was strong, cells were harvested, and DNAs were extracted by the method of Hirai et al. [7]. A fragment including the TK gene of the CE strain, designated PTK, was amplified by polymerase chain reaction (PCR). The primer pair used, designed according to the sequence data of the Thorne strain [2, 6], were: 5' primer: 5' ggtacCAATGGAAGAACAAGTT 3' 3' primer: 5' ggtacCTGGTGAAAGCCACGCTCTCT 3' Restriction endonuclease BamHI sites were added at their 5' ends. The PCR reaction was carried out for 25 cycles with following thermal profile: 94°C (denaturation) for 1 min, 54°C (annealing) for 2 min, 72°C (elongation) for 1 min. The 1.9 kbp PCR product, designated PTK, was purified from 0.8% agarose gel and cloned into the BamHI site of pUC119 (Takara Shuzo Co., Ltd.) (Fig. 1). Escherichia coli strain JM109 was transformed and grown on agar plate in the presence of ampicillin, 5-bromo-4-chloro-3-indyl-[β]-D-galactopyranoside (X-gal; Wako Pure Chemical Industries Ltd.) and isopropyl [β]-D-Thiogalactopyranoside (Wako Pure Chemical Industries Ltd.). White colonies were screened for recombinant plasmids, by a small-scale alkaline lysis plasmid preparation procedure [19]. Insertion of PTK was confirmed by electrophoresis after digestion with BamHI, and the constructed plasmid was designated pPTK.

Insertion vector for the generation of recombinant ILTV was constructed according to the procedure of Sakaguchi et al. [21]. LacZ gene cassette, 4.2 kbp, containing the LacZ gene controlled by the SV40 early promoter, was excised from pCH110 (Pharmacia LKB Biotechnology) by ThhI111 and BamHI digestion and inserted at the SmaI site of the TK gene with a DNA blunting kit (Takara Shuzo Co., Ltd.). Expression of [β]-galactosidase ([β]-gal) of the constructed vector was confirmed by the method of Gerard and Gluzman [5]. Monkey kidney BMT-10 cells, 0.5 × 10⁶, were transfected with 0.5 μg of vector plasmid DNA using 2 μl/ml of Lipofectin reagent (BRL Life Technologies, Inc.) to

detect β-gal activity encoded from LacZ gene. After 2 days, the cells were harvested and frozen and thawed. The thawed cells were centrifuged at 15 Krpm for 5 min and the resulting supernatant was mixed with o-nitro-phenyl-β-D-galactopyranoside (ONPG; Wako Pure Chemical Industries Ltd.) at a final concentration of 50 μg/ml. After incubation at 37°C for 2 hr, the optical density at 420 nm was measured in a spectrophotometer. In result, the vector was confirmed to express the β-gal activity.

CEF was inoculated with 3 × 10⁶ plaque forming units (PFU) of CE strain, and incubated for 7 hr. Cells were trypsinized and mixed with 40 μg of the insertion vector in 0.8 ml of phosphate buffered saline. The cell suspensions were subjected to electrooration at 1,500 V and 25 μF at room temperature with a gene transfer device (Gene Pulser; Bio Rad Co., Ltd.). After the electrooration, the cells were cultured in Eagle’s minimum essential medium (E. MEM) containing 5% fetal calf serum and incubated for 4-6 days at 37°C. To select for the plaques expressing the LacZ gene, the cells were overlayed with E. MEM containing agarose and X-gal at final concentrations of 1% and 0.1 mg/ml respectively. Then the dark blue plaques were picked up with a Pasteur pipette and inoculated onto CEF monolayers, which were cultured at 37°C for several days. The LacZ recombinant ILTV were further purified with plaque isolation.

Southern blot hybridization was carried out to analyse the recombinant ILTV genome. The DNAs of the recombinant virus or parental CE strain were extracted from the infected cells by the method of Hirai et al. [7]. The extracted DNAs were digested with restriction endonuclease HindIII. Since the PTK had no HindIII site, the DNA of the recombinant ILTV was digested with HindIII at the site incorporated in the LacZ gene cassette. These digested samples were electrophoresed through 0.8% agarose gel and transferred to a nylon membrane (Hybond N; Amersham International plc). The LacZ gene and PTK were labelled with digoxigenin using a Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim Biochemica). Hybridization was carried out according to the manufacturer’s protocol. The LacZ probe hybridized to 4.5 kbp and 2.1 kbp fragments of the recombinant virus-DNA, not to parental CE strain DNA (Fig. 2). The PTK probe hybridized to the 2.4 kbp fragment of parental CE strain-DNA and 4.5 kbp and 2.1 kbp fragments of the recombinant virus-DNA (Fig. 2). These results show that the LacZ gene cassette was properly inserted into the TK gene including 2.4 kbp fragment by homologous recombination.

Growth properties of the recombinant virus in CEF were compared with those of the parental CE strain. CEF monolayers were prepared on 10 cm Petri dishes and inoculated with 10⁵ PFU of the recombinant virus or the parental CE strain per dish. After adsorption for 1 hr at 37°C, the monolayers were refilled with E. MEM containing 1% bovine serum and incubated for 6 days at 37°C. One hundred microliters of cultured medium was collected from each dish every 24 hr and the samples were triturated with chicken kidney (CK) cells. Although the growth rate of the recombinant virus in the first four days was less than that of the parental CE strain, the final yield of the recombinant virus was almost the same as that of...
the parental CE strain (Fig. 3).

By means of the TK gene, we showed that ILTV will be one of the vaccine vectors. It is well known that the TK gene is nonessential for herpesvirus growth in tissue culture [4, 11, 18]. However, several reports indicated that the TK gene is important for viral pathogenicity and growth in vivo. For example, Ross et al. showed that the level of growth of TK-inactivated recombinant HVT in vivo appeared to be lower than that of the wild type HVT [20]. It has been shown that TK negative mutants for IBRV [12], PRV [11] and EHV-1 [22] are indeed less pathogenic than the wild type viruses. The recombinant ILTV reported here replicated as well as the parental CE strain did in tissue culture. However, we cannot deduce its level of growth in vivo from the results for the reasons given above. To confirm the feasibility of the recombinant ILTV as a vaccine vector, we have to examine its growth and immunogenicity in chickens.

The LacZ recombinant ILTV that we constructed here could be used to isolate a recombinant ILTV containing a candidate vaccine antigen gene by replacing the LacZ gene. ILTV has several advantages over other poultry viruses as a candidate for a vaccine vector. First, the natural host range of ILTV is limited to a few avian species and does not include mammals. Second, ILTV may be safer than other herpesviruses, because it is not oncogenic. In particular, the CE strain is able to be administered to one-day-old chickens by the s.c. or i.m. route even though they have maternal antibodies to ILTV, and the development of immunity was not hindered as with other ILTV live vaccines by further vaccination with ND-IB live vaccine. Moreover, the CE strain can grow in CEF. This is a very important property because it reduces the cost of production of the ILTV vaccine. This study also suggests the possibility of using the CE strain of ILTV as an available virus vector and its eventual use as a polyvalent vaccine for chickens.

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