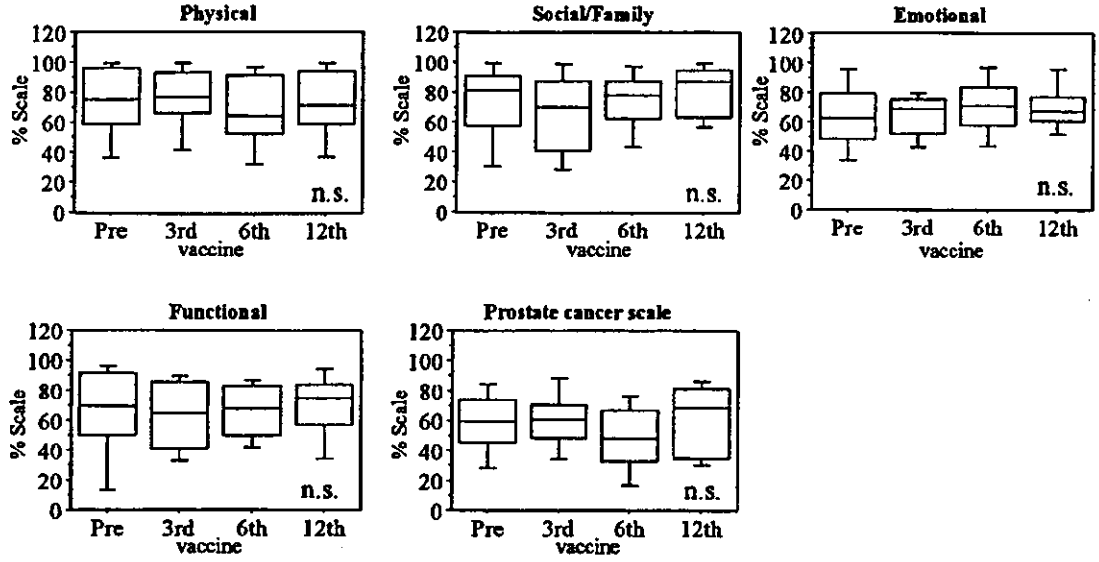


**Fig. 3.** Evaluation of % QOL scales during vaccination. QOL outcomes were separately assessed by the percentage of each scale. ns: not significant.

cancer patients. In the present study, 6 peptides including $PSA_{248-257}$, $PSA_{152-160}$, $PAP_{213-221}$, $PSMA_{624-632}$, $MRP3_{503-511}$, and $MRP3_{1293-1302}$ were added to the previously used 14 candidates for HLA-$A24^+$ patients with HRPC [9]. The most selected peptide in the present study was $PSA_{248-257}$, followed by $PAP_{213-221}$, $SART3_{109-118}$ and $PSA_{152-160}$, while $SART2_{93-101}$, $ART1_{188-196}$, $SART2_{899-907}$, $SART3_{109-118}$, and $Lck_{208-216}$ were selected frequently in the previous study [9]. For specific immunotherapy, it is necessary to determine antigens and epitope peptides that can be recognized by the immune response. We recently reported that both the $PSA_{248-257}$ and $PSA_{152-160}$ peptides are immunogenic in HLA-A24 positive prostate cancer patients, and that the peptide-stimulated

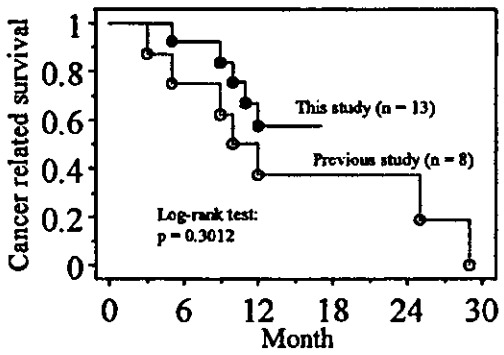


**Fig. 4.** Prostate cancer related survival. There were five observed deaths in the present study group with a median follow-up of 11 months vs. 7 in the previous study group (Noguchi et al., 2003a) with a median follow-up of 13 months. The median survival rate in the present study has not been calculated.

PBMCs of them can show cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner [13]. Inoue et al. revealed that the $PAP_{213-221}$ peptide can induce peptide-specific CTLs from HLA-A2402 positive prostate cancer patients [12]. PSMA is an integral membrane protein and its expression can be detected on prostate carcinoma, even on HRPC [26]. The $PSMA_{624-632}$ peptide was reported to be immunogenic in HLA-A24 positive prostate cancer patients [14]. Also, several antigens, which are not prostate tissue-specific but are highly expressed in prostate cancer, have been identified. Expression of SART2, SART3, and Lck ($p56^{lck}$) proteins was observed in prostate cancer cell line and tissues [9]. Interestingly, in this study, IgGs specific to these CTL epitope peptides were detected in 7 of 14 patients after six vaccinations and 10 of 12 patients during the treatment. These findings suggested these peptides could be recognized by both T-cell and B-cell immunity.

To activate peptide-specific CTLs with peptide vaccine, peptide-pulsed Langerhans cells must come into contact with peptide-specific CTL precursor cells in regional lymph nodes within 2 days [27]. Therefore, determining the frequency of peptide-specific CTL precursors in PBMCs will be important to achieve antitumor immunity. In addition, multiple peptides may be needed to prevent prostate cancer cells from immune escaping. The present approach in immunotherapy for HRPC patients used a new strategy of a pre-vaccination measurement of both humoral and cellular responses to peptides, followed by administration of up to four peptides that had been reactive for pre-vaccination measurement among 16 vaccine candidates (individualized

peptide vaccination). Previous results from a phase I study demonstrated that individualized peptide vaccination was feasible, safe, and immunologically active, but the clinical response has been largely limited [9]. In the present study, all of 16 patients except for one patient also showed an increase of PSA levels during vaccination therapy alone, while augmentation of peptide-specific CTL precursors or peptide-specific IgG was observed in the majority of the patients treated. One of the possible explanations for this discrepancy between immunological and clinical responses is HLA class I antigen down-regulation in prostate cancer. HLA class I antigen down-regulation in prostate cancer may have a negative impact on the outcome of T-cell-based immunotherapy because tumor-specific CTLs failed to show reacting to class I-loss variants. On the contrary, the majority of patients in this study had initially failed to respond to treatments with estramustine phosphate for prostate cancer, whose cells were mostly resistant to estramustine phosphate. Regardless of these factors, the results of the present phase I/II study suggested a benefit by combining individualized peptide vaccination with a low dose of estramustine phosphate in patients with HRPC. The response rate (46%) defined as a serum PSA level decrease of $\geq$50% with a median duration of 7.5 months in the present study is comparable with the response rate in recently reported chemotherapy trials with combinations such as estramustine and paclitaxel (53%) [28], estramustine and docetaxel (62%) [29], and the three-drug combination of estramustine, peclitaxel, and carboplatin (67%) [30]. In other reports, estramustine with paclitaxel and etoposide can prolong the mean survival time (23.4 months), whereas, the regime seems to be accompanied with severe adverse effects [1]. Our results suggested that additive anti-tumor effects could be achieved by the combination of T-cell-based immunotherapy and cytotoxic agents with minimum immunosuppression. In the present study, PSA responses were observed in patients who experienced disease progression prior to estramustine phosphate or peptide vaccination alone, supporting the hypothesis that prostate cancer cells do not have resistance to both vaccination and estramustine phosphate. Cancer cells are extremely robust for their own growth and survival against various perturbations. The highly effective administration of multiple drug regimes can be accomplished only with a system-level analysis of the dynamics of gene regulatory circuits [31]. Further studies on the systems therapy are needed.

The toxicity of the combination regimen reported here was tolerable and this treatment was considered acceptable in the treatment of the vast majority of metastatic HRPC. The most common toxicities were dermatologic reactions at the injection site of the vaccination. There was no hematologic toxicity nor neuropathy reported in estramustine-based or taxane-based chemotherapy regimens for patients with HRPC, and they were dose-limiting toxicities [28–30]. The common toxicities of estramustine treatment include nausea, vomiting, peripheral edema, and vascular events [28]. The incidence of those toxicities in the present study was low because of a low-dose setting of estramustine phosphate. We previously reported that increased humoral response but not cellular response well correlated with overall survival of the vaccinated patients [20]. This issue seemed to be confirmed in this study, although this small scale of the clinical trial alone could not provide any definitive conclusion to a laboratory marker to predict overall survival.

For patients with advanced disease who have reduced life expectancy and no immediate hope for a cure, relief of physical symptoms, and maintenance of function become primary objectives of medical intervention [32]. Systematic symptom assessment may help to clarify a treatment's toxicity, potential palliative benefit, or need to make a change in the patient's clinical management. In the present study, the % QOL scales using FACT-P were stable and well during the treatment, and all patients were treated as out patients.

Based on these findings, we are undertaking larger phase II studies of this regimen.

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# A Randomized Controlled Study of Immunochemotherapy with OK-432 after Curative Surgery for Gastric Cancer

*Yuji Sato,*† Masao Kondo,*† Shigechika Kohashi,*† Norihiko Takahashi,*† Syusaku Takahashi,*† Toshiki Sinohara,*† Ken-ichi Ono,‡ Miyuki Matsuda,‡ Yoshiki Ryoma,‡ Hiroshi Shiroto,† Yukihumi Kondo,† Jun-ichi Uchino,† Kazuo Saito,† and Satoru Todo*†*

**Abstract:** The effect of adjuvant immunochemotherapy including OK-432 (Picibanil) on survival was assessed in patients who underwent curative resection of gastric cancer. Patients enrolled in this randomized controlled study were randomly assigned to group A or group B. Group A patients received 800 mg/d 5'-DFUR (Furtulon) for 2 years from 2 weeks after the operation. Group B patients received OK-432 plus 5'-DFUR by the same regimen as in group A. This study enrolled 288 patients, and 1 patient with malignant lymphoma was excluded. Among the remaining 287 patients, 143 and 144 were allocated to group A and group B, respectively, and their data were included in statistical analysis. The 5-year survival rates for groups A and B were 62.9% and 63.8%, respectively, showing no significant difference ($P = 0.7996$).

**Key Words:** immunotherapy, gastric cancer, randomized controlled study, OK-432

(*J Immunother* 2004;27:394–397)

The prognosis of advanced gastric cancer is not satisfactory, even if macroscopic and pathologic curative resection can be achieved. According to the 44th Report on Gastric Cancer Registration in Japan, the 5-year survival rate was 31.96% in patients with pT3 tumors and 21.05% in those with pT4 tumors.[1] In such patients, recurrence arises mainly in the aortic lymph nodes, liver, lungs, and peritoneum. In patients with scirrhous carcinoma, radical gastrectomy with extended lymphadenectomy and resection of the spleen, colon, and pancreas has been reported to improve the prognosis.[2] However, it has been reported that scirrhous gastric cancer is not indicated for surgery because of its aggressiveness.[3] Chemotherapy, radiotherapy, and immunotherapy have been used in the postoperative treatment of gastric cancer, but their efficacy is unsatisfactory.

OK-432 (Picibanil) is a hemolytic streptococcal preparation approved for the treatment of malignant tumors in 1975.[4] This drug has been used in the treatment of cancer of the gastrointestinal tract, lungs, and head and neck. In the present group study performed by Hokkaido University (Third Trial), the usefulness of chemotherapy combined with subcutaneous OK-432 was assessed in patients who had undergone curative resection of gastric carcinoma.

## MATERIALS AND METHODS

### Eligibility Criteria

Among patients who had undergone macroscopic curative resection and had no macroscopic residual tumor (R0 according to the TNM classification), those who satisfied the following criteria were enrolled in this study. Eligible patients (1) were aged 76 years or younger at the time of surgery, (2) had not received prior radiotherapy, chemotherapy, or immunotherapy, (3) did not have multiple cancer (synchronous or metachronous), (4) were considered fit enough to tolerate surgery and the drugs for this study at the time of preoperative examination, (5) were not allergic to penicillin, (6) had acceptable preoperative laboratory data (white blood cell count $\geq$ 3,000/mm$^3$, platelets $\geq$ 50,000/mm$^3$, Total Protein $\geq$ 5.0 g/dL, creatinine $\leq$ 1.3 mg/dL, and AST/ALT $\leq$ 60 U), (7) had a performance status of 0 to 3, and (8) gave informed consent before surgery.

### Study Procedures

This was a randomized controlled study. Each of the subjects was registered by telephone and was assigned to one of two treatment groups by the central office. A total of 36 institutions (see the appendix) participated in this study, including the First Department of Surgery at Hokkaido University and institutions affiliated with the department.

### Treatments

Patients assigned to group A received 800 mg/d 5'-deoxy-5-fluorouridine (5'-DFUR; Fultulon), a derivative of

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From the *First Department of Surgery, School of Medicine, Hokkaido University, Sapporo, Japan; †Hokkaido University Study Group of Gastric Cancer (Third Trial); and ‡Chugai Pharmaceutical Co., Ltd.

Reprints: Yuji Sato, MD, First Department of Surgery, School of Medicine, Hokkaido University, N-14, W-5, Kita-ku, Sapporo 060-8648, Japan (e-mail: yuchan@med.hokudai.ac.jp).

TABLE 1. Background Characteristics

| Characteristics | Group A (Control Group) | Group B (OK-432 Group) | Chi-Square *P* Value | Characteristics | Group A (Control Group) | Group B (OK-432 Group) | Chi-Square *P* Value |
|---|---|---|---|---|---|---|---|
| Gender | | | | pT | | | |
| Male | 100 | 103 | 0.766 | pT1 | 25 | 23 | 0.870 |
| Female | 43 | 41 | | pT2 | 87 | 84 | |
| Age | | | | pT3 | 28 | 34 | |
| 20–29 | 1 | 1 | 0.643 | pT4 | 3 | 3 | |
| 30–39 | 1 | 3 | | pN | | | |
| 40–49 | 10 | 11 | | pN0 | 52 | 52 | 0.849 |
| 50–59 | 37 | 29 | | pN1 | 45 | 50 | |
| 60–69 | 53 | 64 | | pN2 | 36 | 35 | |
| 70–76 | 41 | 36 | | pN3 | 8 | 5 | |
| Macroscopic type | | | | pN4(M1) | 2 | 2 | |
| Superficial | 16 | 9 | 0.263 | pTNM | | | |
| Borrman 1 | 8 | 5 | | IA | 17 | 15 | 0.514 |
| Borrman 2 | 47 | 40 | | IB | 35 | 35 | |
| Borrman 3 | 39 | 41 | | II | 41 | 37 | |
| Borrman 4 | 9 | 13 | | IIIA | 26 | 37 | |
| Unclassified | 23 | 35 | | IIIB | 18 | 18 | |
| Histologic type | | | | IV | 6 | 2 | |
| pap | 7 | 2 | 0.743 | Lymph node dissection | | | |
| tub1 | 21 | 19 | | D1 | 5 | 3 | 0.597 |
| tub2 | 34 | 33 | | D2 | 111 | 118 | |
| por | 58 | 65 | | D3 | 27 | 23 | |
| muc | 5 | 4 | | | | | |
| sig | 9 | 10 | | | | | |
| ud | 2 | 1 | | | | | |
| others | 7 | 10 | | **Total** | 143 | 144 | |

The two groups were not significantly different with respect to gender, age, macroscopic tumor type, histologic tumor type, pT, pN, pTNM stage, and lymph node resection.

pap, papillary adenocarcinoma; tub1, tubular adenocarcinoma well-differentiated type; tub2, tubular adenocarcinoma moderately differentiated type; por, poorly differentiated type; muc, mucinous adenocarcinoma; sig, signet-ring cell carcinoma; ud, undifferentiated carcinoma.

5-fluorouracil,[5,6] for 2 years from 2 weeks after surgery. Those assigned to group B received 5'-DFUR by the same regimen as well as subcutaneous OK-432.[4] The initial dose of OK-432 was 3 KE, which was increased to 5 KE at maximum. One KE corresponds to 0.1 mg dried streptococcal cells. OK-432 (3–5 KE) was given at an interval of 7 to 14 days. The patients were administered both chemotherapy and OK-432 for 2 years.

## Outcome Measures

Survival at 5 years after surgery was the end point of this study. We also assessed the effect of OK-432 on the 5-year survival rate. The survival time was calculated from the day of operation. Adverse events were compared between the two groups.

## Target Number of Patients

The number of patients to be recruited was determined as follows. If 55% of the patients receiving chemotherapy alone are alive 5 years after surgery and if the 5-year survival rate increases by 15% when OK-432 is administered together with chemotherapy, the number of patients per group needs to be 135 at an $\alpha$ error of 0.05 and $\beta$ error of 0.80.[7] Consequently, we planned to recruit 300 patients (150 per group) for this study.

## Statistical Analysis

To compare the two groups in terms of background characteristics, the chi-square test was used. The cumulative survival rate was calculated by the Kaplan-Meier method. The log-rank test was used to compare the survival rates for groups A and B. $P < 0.05$ was considered significant, and $P < 0.10$ was

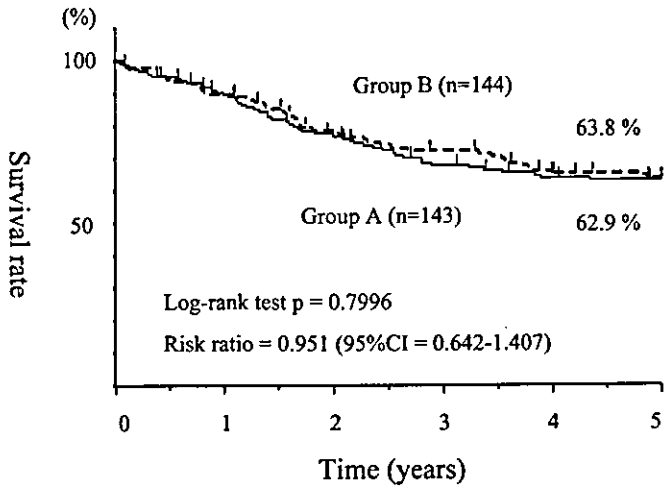


**FIGURE 1.** Overall survival of eligible patients. The 5-year survival rates for groups A and B were 62.9% and 63.8% respectively, showing no significant difference ($P = 0.7996$, risk ratio = 0.951, 95% confidence interval = 0.642–1.407).

nearly significant. The Cox proportional hazards model[8] was used to calculate risk ratios and 95% confidence intervals. All statistical analyses were performed with SAS software version 6.12 (SAS Institute, Cary, NC).

## RESULTS

### Patients

A total of 288 patients were registered during the 26-month period from January 1, 1992, to March 31, 1994. The number of patients registered per institution ranged from 1 to 24, with a mean of 11.1. Of the 288 patients, 1 with malignant lymphoma in group B was considered ineligible. In accordance with the intent-to-treat principle, data on the remaining 287 patients (excluding the 1 ineligible case) were subjected to analysis (143 and 144 patients in groups A and B, respectively).

### Background Characteristics

Group A included 100 men and 43 women, Group B 103 men and 41 women. The patients were 20 to 76 years old (mean 49.8 years in group A and 50.2 years in group B). The two groups were not significantly different with respect to gender, age, macroscopic tumor type, histologic tumor type, and pT, pN, or pTNM stage (Table 1).

### Survival Rate

The survival status at 5 years after surgery could be determined for all eligible patients (n = 143 and 144 in groups A and B, respectively). The 5-year survival rates for groups A and B were 62.9% and 63.8%, respectively, showing no significant difference ($P = 0.7996$, risk ratio = 0.951, 95% confidence interval = 0.642–1.407) (Fig. 1).

### Adverse Events

Mild fever and injection site reactions were the only adverse events caused by OK-432 (Table 2). None of these events required any specific treatment.

## DISCUSSION

The hemolytic streptococcal preparation, OK-432, was developed by Okamoto et al[4] as an agent that could activate cellular immunity.[9,10] It has also been reported that OK-432 is effective for immunochemotherapy after curative resection.[11–13] However, other studies found that there was no improvement attributable to OK-432 when it was used in combination with chemotherapy,[14] so the usefulness of OK-432 has not yet been established. It is rare for the effectiveness of a drug to be established by a single clinical study. In recent years, the effectiveness of drugs has been assessed by meta-analysis.[15] We performed a study with Sakamoto et al[16] to assess the effectiveness of OK-432 combined with chemotherapy after curative resection in 1,522 patients with gastric cancer. The 3-year survival rates for patients receiving chemotherapy with and without OK-432 were 67.5% and 62.5%, respectively, so OK-432 seemed to improve the response to postoperative chemotherapy. The patients had been followed for only 3 years at the time our previous report was prepared. To allow meta-analysis of the data, the patients who were alive at 3 years after surgery were traced to determine their survival status at 5 years postoperatively and to calculate the 5-year survival rates. Individual patient data meta-analysis, with the endpoint being set as survival status 5 year after surgery, is in progress.

**TABLE 2.** Adverse Events

| Adverse Event | Group A (Control Group) | Group B (OK-432 Group) |
|---|---|---|
| Leukocytes | 8 | 6 |
| Hemoglobin | 2 | 2 |
| Platelets | 2 | 1 |
| Anorexia | 9 | 15 |
| Diarrhea | 7 | 10 |
| Hepatic | 1 | 3 |
| Eczema | 5 | 4 |
| Stomatitis | 1 | 0 |
| Fever | 0 | 20 |
| Injection site reaction (pain and redness) | 0 | 4 |

Mild fever and injection site reactions were the only adverse events caused by OK-432. None of these events required any specific treatment.

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## APPENDIX

Participating institutions: Hokkaido University, Sapporo National Hospital, West Sapporo National Hospital, Ashibetu Municipal Hospital, Iwamizawa Municipal Hospital, Otaru Municipal Hospital, Sapporo Municipal Hospital, Shibetu Municipal Hospital, Sunagawa Municipal Hospital, Chitose Municipal Hospital, Tomakomai Municipal Hospital, Bibai Municipal Hospital, Fukagawa Municipal Hospital, Mikasa Municipal Hospital, Yubari Municipal Hospital, Wakkanai Municipal Hospital, Nakashibetu Town Hospital, Asahikawa Kosei General Hospital, Abashiri Kosei General Hospital, Engaru Kosei General Hospital, Sapporo Kosei General Hospital, Obihiro Kyoukai Hospital, Hakodate Kyoukai Hospital, Furano Kyoukai Hospital, Konan Hospital, Sapporo Social Insurance General Hospital, Hokkaido Central Hospital, Kushiro Rosai Hospital, Nikko Memorial Hospital, Tenshi Hospital, Minami Ichijyou Hospital, Sapporo Hokuyu Hospital, Kaisei Hospital, Kawanishi Hospital, Megumino Hospital, Keiwakai Ebetu Hospital.

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# Development of Improved Sindbis Virus-Based DNA Expression Vector

RYUYA YAMANAKA[1,2] and KLEANTHIS G. XANTHOPOULOS[1]

## ABSTRACT

**We have constructed an improved DNA expression vector based on the Sindbis virus. Several DNA-based Sindbis virus vectors were constructed to investigate the efficiency of transgene expression. These vectors, when transfected into mammalian cells, have been used to express heterologous genes. A recombinant genome of Sindbis plasmid DNA, in which the structural genes were replaced by a polylinker cassette to allow for insertion of heterologous genes, was placed under the control of a simian virus (SV 40) promoter with a hepatitis delta virus (HDV) antigenomic ribozyme and a polyadenylation signal. Transfection of mammalian cells with this Sindbis-based plasmid vector, pSin-SV40-HDV-SV40pA, resulted in transient high-level expression of the β-galactosidase reporter gene. The expression level of β-galactosidase from pSin-SV40-HDV-SV40pA was more than 16-fold higher than that of pSin-Lux originally reported by Herweijer *et al.* *In vivo* expression was also detected after injection of plasmid DNA into mouse quadriceps. *In vivo* expression was transient and undetectable after day 14. Furthermore, we demonstrate that the transfection of cells with this Sindbis virus vector results in apoptotic death on glioma cells. We have demonstrated a high-level expression of the exogenous β-galactosidase gene from the pSin-SV40-HDV-SV40pA construct using a Sindbis replication system.**

## INTRODUCTION

GENE TRANSFER APPLICATIONS have been hampered by very low transfection efficiency and low expression levels. To increase gene expression levels, several self-amplifying systems have been developed (Hahn *et al.*, 1992; Deng and Wolff, 1994; Herweijer *et al.*, 1995; Yamanaka *et al.*, 2001a,b). Sindbis virus, a member of the Alphavirus, has received considerable attention for use as a virus-based expression vector. The Sindbis virus expression system differs from currently available viral delivery systems in that it is an RNA virus, known to generate high levels of protein expression *in vitro*. The Sindbis virus is less pathogenic (Piper *et al.*, 1994) to humans. The Sindbis virus is a self-amplifying expression vector, production of high titered stocks of infectious particles, infection of nondividing cells, generating high-level expression of transgenes (Strauss and Strauss, 1994). After infection of a host cell, the RNA genome functions as a mRNA for the translation of the nonstructural proteins. These subsequently replicate the virus by copying the (+)-strand RNA genome into the (−)-strand RNA and vice versa. Transcription starting at the internal subgenomic promoter present in the (−) strand results in the production of large amounts of the subgenomic mRNA (Levis *et al.*, 1990; Herweijer *et al.*, 1995). In these systems, the subgenomic RNA sequence coding for the structural proteins has been replaced by the transgene sequence of interest (Bredenbeek *et al.*, 1993).

Herweijer *et al.* (1995) reported a plasmid-based self-amplifying Sindbis virus vector, pSin-Lux. We have generated improved the Sindbis virus vector system, working more efficiently than pSin-Lux. In this report, we describe the development of a Sindbis virus-derived DNA-based expression vector which can initiate the replication cascade in transfected mammalian cells to produce high-level expression of a reporter gene. Furthermore, we demonstrate that the transfection of cells with this Sindbis virus vector results in cell death.

[1]Clinical Gene Therapy Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland.
[2]Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata, Japan.

## MATERIALS AND METHODS

### *Plasmid DNA construction*

For the construction of plasmid DNA vectors, pSinRep 6 containing an Eco47III, Sma I, and Sst I site at the Sindbis nsp 5′ end was created from pSinRep 5 (Invitrogene, San Diego, CA) by overlapping PCR with the primers RY204: GGGGGAGCTCCCCGGGATTGACGGCGTAGTACACA, and RY205: GGGAGCGCTGCTAAAAGAGGCTGGGACTT. The Sst I and Eco47III site was included at the 5′-end forward and reverse primers, respectively, to facilitate insertion into the pSinRep 5 plasmid. The CMV/IE, SV 40, and RSV promoters were inserted into pSinRep 6 between the unique Sma I site. The CMV/IE enhancer/promoter, RSV promoter, SV 40 promoter sequence, and the SV40 polyadenylation signal sequence were obtained by polymerase chain reaction using the pRL-CMV, pRL-SV40 (Promega, Madison, WI), and pSin-Lux as a template. The primers used were RY215: AAAAACCCGGGGGATCCGGCCATTAGCCATA, RY216: AAAAACCCGGGCGATAAGCTTGGGGCCGCGG for CMV, RY217: GGGGGGAGCTCGTCGACCAATTCTCATGTTT, RY218: AAAAACCCGGGGCTAGCAAGCTTGCTAGCAGCT for RSV and RY209: GGGGGCCCGGGAATGTAACTGTATTCAGCGA, RY210: GGGGGCCCGGGGGATCCAGACATGATAAGA for SV40. The SV 40 poly (A) was fused to the vector 3′ end, at Sindbis virus nt 8067. The hepatitis delta virus (HDV) antigenomic ribozyme (Perrotta and Been, 1991) was inserted between the 3′ sindbis end and SV40 polyadenylation signal. The HDV ribozyme sequence, with Sac I sites at each end, was generated. HDV double-strand DNA was constructed using oligonucleotide RY230: GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCC GAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAG and an antisense one. Correct- and reverse-sense HDV insertions were verified by sequence analysis. Additional plasmids were constructed to compare expression levels with Sindbis virus expression vectors. The β-galactosidase gene was positioned in the polylinker cassette. A total of nine plasmids were generated according to the combination of promoters, HDV, and SV 40 poly (A). All of these construct were confirmed by sequence analysis. These constructs were designated as shown in Figure 1. pSin-Lux was a generous gift from Dr. Jon A. Wolff. The β-galactosidase gene was also inserted instead of the luciferase gene. The construct was designated as pSin-LacZ. β-Galactosidase activity of pSin-LacZ was 2.0 ± 0.2. The standard deviation represents six samples.

### *β-Galactosidase activity measurement* in vitro

To measure β-galactosidase activity, $1 \times 10^6$ baby hamster kidney cells (BHK) were cotransfected with 1 μg of each plasmid by lipofection (Invitrogene), and β-galactosidase activity was measured in cell lysates at 48 h after DNA transfection using a β-galactosidase assay kit (Promega, Madison, WI). pBlueScript II Phagemid vector (pBS: Stratagene, La Jolla, CA) was used as a control vector.

### *β-Galactosidase activity measurement* in vivo

Expression of β-galactosidase activity in C57BL/6 mice quadriceps as described previously (Dubensky *et al.*, 1996).

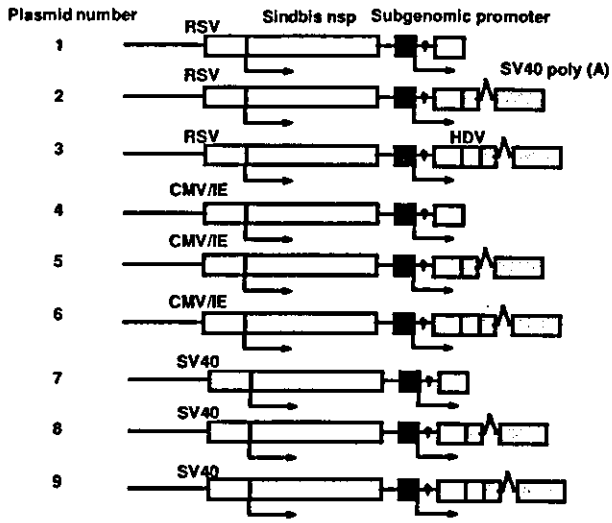


**FIG. 1.** A schematic of Sindbis virus vectors used in this study. Arrows indicate the start site of transcription. RSV, Rous sarcoma virus long terminal repeat; CMV/IE, human cytomegalovirus immediate early promoter; SV 40, simian virus 40 promoter; Sindbis nsp, Sindbis virus sequences coding for the nonstructural proteins; SV40 poly (A), simian virus 40 termination/polyadenylation signal; HDV, hepatitis delta virus antigenomic ribozyme.

Plasmid DNA was injected into mouse quadriceps (10 μg of DNA in 100 μl of saline per leg). Mice were sacrificed and quadriceps were removed, β-galactosidase extracts from excised muscles were made by homogenization (Pro200, Pro Scientific Inc., Monroe, CT) in 1 ml of buffer, and assayed for β-galactosidase expression at several time points after injection of DNA. Six quadriceps were injected and assayed per time point.

### *Tumor cell lines and animal models*

Baby hamster kidney (BHK) cells were obtained from Riken Cell Bank (Tsukuba, Japan). A172 and MO59K cell line were from ATCC (Manassas, VA). Cell lines were grown in MEM medium (GIBCO BRL, Gaitherburg, MD) containing 10% FCS. All cell lines were shown to be free from Mycoplasma contamination. All experiments used 5–8-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), which were maintained in a virus-free environment and treated in accordance with the NIH Laboratory Animal Resources Commission standards.

## RESULTS

### *The combination of SV40 promoter, HDV ribozyme, and SV40 poly (A) cassette generated most efficient gene expression (Fig. 1 and Table 1)*

Plasmid pSinRep5 (Invitrogene), a Sindbis virus-based plasmid vector, contains ns Ps genes followed by a subgenomic RNA promoter and a polylinker cassette, which replaces the structural genes of the Sindbis virus vector. The ns Ps genes

TABLE 1. EXPRESSION OF β-GALACTOSIDASE ACTIVITY IN PLASMID VECTORS

| *Plasmid number* | *Plasmid name* | *β-Galactosidase activity (mU/μg protein)* |
|---|---|---|
| 1 | pSin-RSV | 2.0 ± 0.2 |
| 2 | pSin-RSV-SV40 | 2.2 ± 0.2 |
| 3 | pSin-RSV-HDV-SV40 | 2.2 ± 0.3 |
| 4 | pSin-CMV/IE | 5.0 ± 0.8 |
| 5 | pSin-CMV/IE-SV40 | 6.4 ± 0.7 |
| 6 | pSin-CMV/IE-HDV-SV40 | 8.3 ± 0.8 |
| 7 | pSin-SV40 | 10.0 ± 0.9 |
| 8 | pSin-SV40-SV40 | 15.2 ± 1.2 |
| 9 | pSin-SV40-HDV-SV40 | 32.5 ± 2.5 |

Shown on the left side of Fig. 1 and adjacent to each vector are the levels of β-galactosidase detected, in mU per μg of protein.

are under the transcriptional control of the SP6 promoter; therefore, *in vitro* transcription and capping of the 5′ end are required for the expression of the gene of the interest in mammalian cells. To place pSinRep 5 under the control of a RNA polymerase II promoter, a cytomegalovirus (CMV) immediate-early (IE) enhancer/promoter, a simian virus (SV) 40 promoter sequence, and Rouse sarcoma virus (RSV) promoter was inserted upstream of the ns P1 gene, respectively. In addition, to ensure that mRNA transcribed from the plasmid was properly terminated, the SV 40 late polyadenylation signal sequence was inserted downstream of the polylinker cassette. Moreover, the hepatitis delta virus (HDV) antigenomic ribozyme was inserted upstream of transcriptional/polyadenylation signal. A total of nine constructs with a combination of these sequence were obtained, as shown in Figure 1. Transcript 5′-end mapping in cells transfected with Sindbis DNA vectors was investigated. The results of this study demonstrated that the primer extension products of RNAs isolated from cells transfected with pSin-RSV, pSin-CMV/IE, or pSin-SV40 were the same lengths (data not shown). The transcriptional start site shoud be the same for the nine constructs. The expression level of β-galactosidase obtained following transduction of pSin-SV40-HDV-SV40pA was 32.5 ± 2.5 mU/μg protein; on the other hand, that of the pSin-LacZ was 2.0 ± 0.2 mU/μg protein. The expression level of β-galactosidase obtained following transduction of pSin-SV40-HDV-SV40pA was more than 16-fold higher than that obtained with the pSin-Lux (Lac Z) originally reported by Herweijer *et al.* (1995). The combination of the SV40 promoter, HDV ribozyme, and the SV40 poly (A) cassette generated most efficient gene expression, as shown in Table 1.

### *Expression levels for the Sindbis vector peaked earlier in time, and were approximately 30-fold higher compared to the pBS cassette (Table 2)*

β-Galactosidase expression after transfection of the Sindbis expression vector pSin-SV40-HDV-SV40pA into BHK 21 cells was measured at several days after transfection. As shown in Table 2, the results are compared to cells transfected with pBS. Expression levels for the Sindbis vector peaked 2 days after and were approximately 30-fold higher compared to the pBS cassette. The Sindbis expression plasmids were also tested in human A172 and MO59K glioma cells. β-Galactosidase expression measured after transfection of pSin-SV40-HDV-SV40pA was comparable in these cells (data not shown).

### *Apoptosis was induced after transfection of pSin-SV40-HDV-SV40pA (Fig. 2)*

Approximately 72 h following the transfection, about 80% of the cells tranfected with pSin-SV40-HDV-SV40pA showed a condensed round form and dense blue color as shown in Figure 2. Apoptosis was induced, resulting from caspase-3 ac-

TABLE 2. EXPRESSION OF β-GALACTOSIDASE ACTIVITY *IN VITRO*

| *Days after injection* | *pSin-SV40-LacZ-HDV-SV40pA* | *pBS* | |
|---|---|---|---|
| 1 | 21.0 ± 4.0 | 1.0 ± 0.2 | $P < 0.01$ |
| 2 | 30.0 ± 5.5 | 1.0 ± 0.3 | $P < 0.01$ |
| 3 | 20.0 ± 4.5 | 2.0 ± 0.4 | $P < 0.01$ |
| 4 | 16.0 ± 3.5 | 1.5 ± 0.2 | $P < 0.01$ |
| 5 | 9.5 ± 1.5 | 1.5 ± 0.3 | $P < 0.01$ |

One microgram of plasmid DNA was transfected into BHK cells grown in 35-mm dishes. β-Galactosidase activity was measured at various days following transfection. Three dishes were measured per time point. Similar results were obtained in two other experiments.

A more than 30-fold higher value was obtained by pSin-SV40-HDV-SV40pA than pBS after 2 days of transfection. Plasmid pBS was used as a positive control in this experiment. Student's *t*-test was used for calculating the significance of each data. Statistical significance was determined at the <0.05 level.

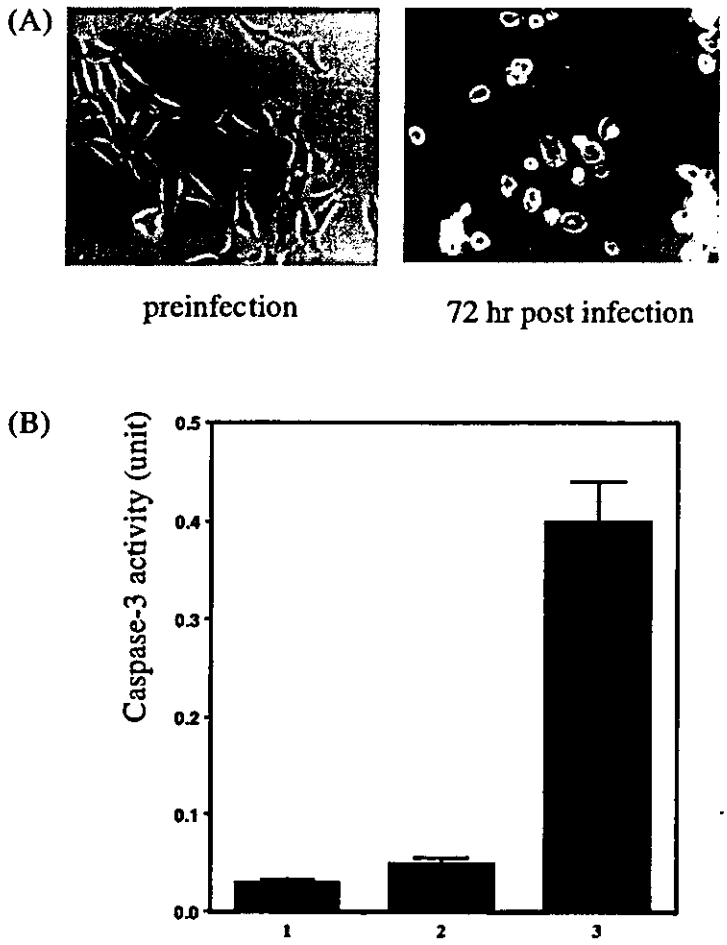


**FIG. 2.** Induction of apoptotic death of host cells by self-replicating Sindbis DNA vector. Apoptotic cell death was observed 72-h posttransfection on A172 glioma cells (Riken cell bank, Tsukuba, Japan) (A). Caspase 3 activity was enhanced 72-h posttransfection on A172 glioma cells by pSin-SV40-LacZ-HDV-SV40 (B). Caspase 3 activity was measured using caspase detection system (Promega, Madison, WI). Transfection by no plasmid (1), pBS (2) and pSin-SV40-LacZ-HDV-SV40 (3).

tivity upregulation after transfection of pSin-SV40-HDV-SV40pA. After being transfected with pSin-SV40-LacZ-HDV-SV40pA, cells rounded up, shrunk, and stopped dividing after 24 h. At 48 h, these cells had the nuclear fragmentation characteristic of apoptosis. Nearly all of the cells died by 96 h. Caspase-3 activity was a 0.4 ± 0.05 unit, 72 h posttransfection on A172 glioma cells by pSin-SV40-LacZ-HDV-SV40 compared to 0.06 ± 0.02 by pBS. Protein synthesis in host cells infected by wild-type Sindbis virus is inhibited by the competitive production of viral proteins, consequently leading to cell death.

TABLE 3. EXPRESSION OF β-GALACTOSIDASE ACTIVITY *IN VIVO*

| *Days after injection* | *pSin-SV40-LacZ-HDV-SV40pA* | *pBS* | |
|---|---|---|---|
| 2 | 15.5 ± 2.0 | 2.5 ± 0.3 | $P < 0.01$ |
| 4 | 32.0 ± 5.5 | 3.0 ± 0.3 | $P < 0.01$ |
| 7 | 12.0 ± 2.0 | 4.5 ± 0.5 | $P < 0.01$ |
| 10 | 6.5 ± 1.5 | 4.0 ± 0.6 | |
| 14 | 4.0 ± 1.0 | 5.5 ± 0.7 | |

Expression of β-Galactosidase activity in C57BL/6 mice (5–8 weeks old) quadriceps. Plasmid DNA was injected into mouse quadriceps. Six quadriceps were injected and assayed per time point. β-Galactosidase expression from pBS increase during the first 3 weeks after injection; in contrast, expression from pSin-SV40-HDV-SV40pA was maximum at day 4 after injection and diminished rapidly at later time points. At day 14, very low level expression could be detected. Student's *t*-test was used for calculating the significance of each data. Statistical significance was determined at the <0.05 level.

*Expression appears to peak much earlier and diminished rapidly (Table 3)*

In the *in vivo* experiment, β-galactosidase expression from pBS increased during the first 2 weeks after injection; in contrast, expression from pSin-SV40-HDV-SV40pA was maximum at day 4 after injection and diminished rapidly at later time points; at day 14, a very low-level expression could be detected, as shown in Table 3. This transient nature *in vivo* could allow for safe, short-term delivery of gene product. Also, expression appears to peak much earlier, which could be an additional benefit for vaccination approaches.

## DISCUSSION

The Sindbis virus vector is being developed as a vector for expression of heterologous genes, and has many advantages for the expression vector systems. In the Sindbis virus vector systems, because helper RNA does not contain a packaging signal, it will not form a defective interfering particle or be packaged with recombinant RNA. Furthermore, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule, without a DNA intermediate (Strauss and Strauss, 1994). This is in contrast with retroviruses, which must enter the nucleus and integrate into the host genome for initiation of the vector activity. Thus, retrovirus vectors have applications for long-term expression of foreign genes, while the Sindbis virus vector is useful primarily for transient high-level expression. Furthermore, although adenovirus vectors can express high levels of foreign genes, these systems are more complex than the Sindbis virus vector, and express many highly antigenic virus-specific gene products including structural proteins (Rosenfeld *et al.*, 1991). In contrast, the current Sindbis virus vector, express only the four viral replicase proteins (ns P 1–4) required for RNA amplification in the transduced cells.

Herweijer *et al.* (1995) and Dubensky *et al.* (1996) independently constructed plasmid vectors from the Sindbis virus. Herweijer *et al.* (1995) reported the usefulness of the pSin-Lux plasmid. This is under the transcriptional control of the RSV promoter. The expression level generated by the pSin-Lux vector was up to 200 times higher than those obtained with a conventional RSV expression vector. *In vivo* expression was transient and undetectable by day 16. In our experiment, the expression level of β-galactosidase from pSin-SV40-HDV-SV40pA was more than 16-fold higher than that of the pSin-Lux originally reported by Herweijer *et al.* (1995). Dubensky *et al.* (1996) found the CMV promoter was two- to fourfold better than the RSV they used. They also reported a three- to fourfold increase with the two promoters when the cDNA contained the HDV ribozyme. Kohno *et al.* (1998) constructed plasmid vectors from Semliki Forest virus, a subgroup of Alphavirus with three envelope proteins, compared with the Sindbis virus, which has two envelope proteins. In their systems as well, they showed 10–30-fold higher expression compared to conventional plasmid cassette. Our system has much more efficient gene expression of an exogenous gene than those reported self-amplifying systems. In this study, for the RSV promoter, the addition of the SV40 polyA sequence or HDV ribozyme had no effect. With the CMV promoter, the differences were minor. With the SV40 promoter, however, the difference was as much as threefold. The combination of the SV 40 promoter, SV40 polyA sequence, and HDV ribozyme were much more efficient for the Sindbis DNA expression system.

A major advantage of the Sindbis virus-derived plasmid vector, pSin-SV40-HDV-SV40pA, is a high level expression of an exogenous gene using the self-amplifying systems of the Sindbis virus. In addition, this vector is transfected into cells as double-stranded DNA. Therefore, there is no need for *in vitro* transcription and mRNA capping as required for the transfection of previously described Sindbis virus-derived RNA vectors. A DNA copy of a recombinant Sindbis virus was placed under the transcriptional control of a RSV, CMV, and SV40 promoter. Upon introduction of this plasmid into mammalian cells, the full-length (+)-strand RNA genome is transcribed from this promoter. Transport of this transcript to the cytoplasm started the self-amplification and transgene expression. Self-amplifying vectors only need a single RNA copy to reach the cytoplasm, they can generate a high expression level almost independent of initial transfection and transcription efficiency.

We have reported that the Semliki Forest virus, the Alpha virus family resembling the Sindbis virus, has been powerful tools in inducing anti-tumor immunity for brain tumors (Yamanaka *et al.*, 2001a, 2002a; 2002b). We have proposed that intratumorally injected transduced immature DCs can acquire and process tumor antigens *in situ*, migrate to lymphoid organs, and then initiate a significant tumor-specific immune response. Thus, the self-replicating Alpha virus system could serve as a powerful tool in treating malignant tumors. The Sindbis virus vector described here is plasmid based and is self-initiating. The RNA virus is manipulated and transfected into cells as a double-stranded DNA plasmid. Thus, the self-replicating Alpha virus system could serve as a powerful tool in gene therapy.

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Address reprint requests to:
*Ryuya Yamanaka, M.D.*
*Department of Neurosurgery*
*Brain Research Institute*
*Niigata University*
*Asahimachi-dori 1-757*
*Niigata City, Japan 951-8122*

*E-mail:* ryaman@bri.niigata-uac.jp

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