Cytolytic Activity Induced by Intramuscular Injection of Plasmid DNA Expressing the Nucleocapsid Protein of the JHM Strain of Mouse Hepatitis Virus into C57BL/6 Mice

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ABSTRACT. We constructed plasmids expressing mRNA7 coding the nucleocapsid (N) protein of JHM strain of MHV (JHMV) under the control of Rous sarcoma virus (RSV) LTR or human elongation factor (EF) 1a promoter, referred to as pRSV-mRNA7 and pEF-mRNA7, respectively. Although only a slight level of cytolysis was observed by the spleen cells from C57BL/6 mice injected intramuscularly with pEF-mRNA7, the spleen cells from the mice administered with pRSV-mRNA7 showed a significant level of cytolytic activities against the cells expressing the viral N protein. The difference in the level of specific cytolysis might have been mainly due to a difference in the expression levels of the N protein in the muscles between the mice injected with pEF-mRNA7 and pRSV-mRNA7, since the specific activity of chloramphenicol acetyltransferase (CAT) in muscles from the mice injected with plasmid DNA expressing CAT gene directed by RSV LTR was significantly higher than that in those administered by the plasmid DNA directed by EF-1a promoter. — KEY WORDS: cytolytic activity, i.m. injection, mouse hepatitis virus, nucleocapsid protein, plasmid DNA.

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Mouse hepatitis virus (MHV) is a natural pathogen of mice which causes a variety of diseases, including hepatitis and encephalitis [3, 12]. Infection of susceptible strains of mice with the neurotropic JHM strain of MHV (JHMV) produces a panencephalitis accompanied by primary demyelination with no or little evidence of hepatitis. This infection is studied as a model of virus-induced central nervous system (CNS) demyelination [3, 5, 23]. The immune response during the acute phase of the disease appears to play a pivotal role in determining the eventual outcome of the infection [3, 12, 26]. The major components of the immune system, including the antiviral antibody, natural killer cells, monocytes and $CD4^+$ and $CD8^+$ T cells, are present within the CNS during the acute infection [12, 26, 27]. However, their respective roles in protection from the acute infection and in the chronic disease remain unclear. The adoptive transfer of virus-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) clones not only protects mice from a lethal JHMV infection but also reduces virus replication within the CNS, thereby providing some measure of protection from chronic disease [30]. CTLs recognize peptides derived from immunogenic proteins in association with major histocompatibility complex (MHC) class I molecules [2, 13]. Stohlman *et al.* [22] showed that immunization of BALB/c mice (H-2^d) with JHMV induces CTLs that are restricted to the L^d class I molecule and are specific for the nucleocapsid (N) protein. The N protein is a basic, highly phosphorylated internal virus structural protein that forms a helical nucleocapsid in association with the 32 kilobase genomic RNA [13].

Recently, it has been reported that intramuscular (i.m.) injection of DNA expression vectors in mice results in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA [1, 28]. Plasmids are shown to be maintained episomally and do not replicate. Persistent expression is observed after i.m. injection in skeltal muscle of rats and primates, and in cardiac muscle of rats [10, 14, 29]. This technique can be a method of introducing viral proteins into the antigen-processing pathway that results in the generation of virus-specific CTLs [17, 25, 29].

To investigate the role of CTLs in protection against acute infection and chronic disease caused by JHMV, we first examined the induction of cytolytic activities by i.m. injection of the plasmid that expressed the N protein of JHMV into C57BL/6 mice (H-2^b).

MATERIALS AND METHODS

Cells and virus: Murine astrocytoma-derived DBT cells [9] were cultured in Eagle's minimum essential medium (MEM) with 5% calf serum (CS) at 37°C in an atmosphere of 5% CO_2. C57BL/6 mouse fibrosarcoma-derived QR32 cells [19] were maintained in Eagle's MEM supplemented with 8% fetal calf serum (FCS), 1 mM sodium pyruvate, non-essential amino acids (Gibco) and 2 mM L-glutamine. The JHM strain of MHV (JHMV) [16] was propagated in DBT cells.

Mice: Pathogen-free C57BL/6//JCL (C57BL/6) mice (H-2^b) were purchased from CLEA, Japan at 4 weeks of age. Serum samples obtained from representative mice were tested for JHMV specific antibody by enzyme-linked immunosorbent assay (ELISA) and found to be negative. Research was conducted according to the principles defined in the "Guide for the Care and Use of Laboratory Animals" prepared by Rakuno Gakuen University.

Construction of plasmids: A full length of cDNA

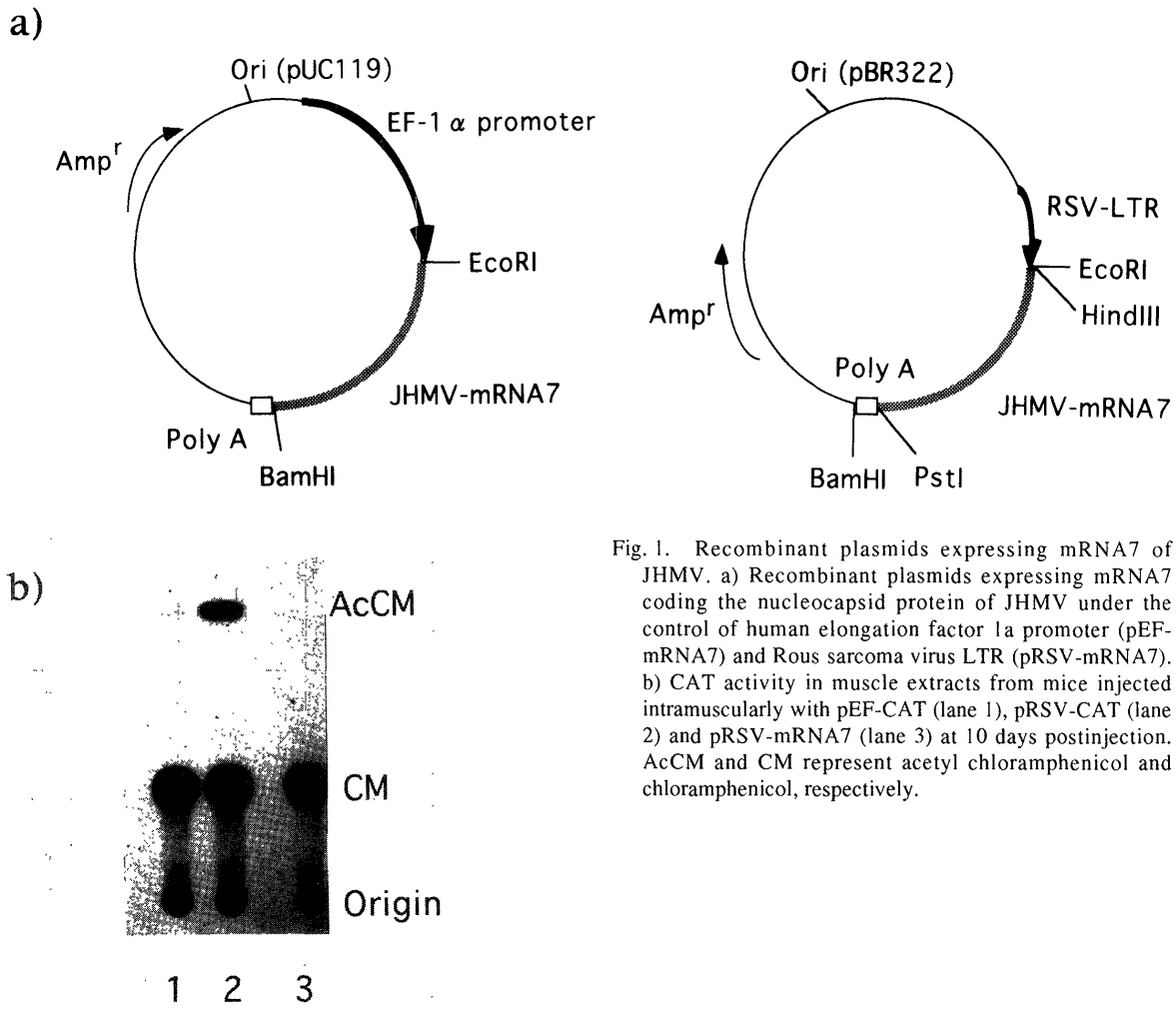

Fig. 1. Recombinant plasmids expressing mRNA7 of JHMV. a) Recombinant plasmids expressing mRNA7 coding the nucleocapsid protein of JHMV under the control of human elongation factor 1a promoter (pEF-mRNA7) and Rous sarcoma virus LTR (pRSV-mRNA7). b) CAT activity in muscle extracts from mice injected intramuscularly with pEF-CAT (lane 1), pRSV-CAT (lane 2) and pRSV-mRNA7 (lane 3) at 10 days postinjection. AcCM and CM represent acetyl chloramphenicol and chloramphenicol, respectively.

fragment of mRNA7, which codes the N protein of JHMV [21], was ligated into the expression vector pEF-321T [11] after removal of the SV40-large T gene by digestion with *Hin*dIII-*Hpa*I and formation of a blunt-end with a fill-in reaction. The cDNA of mRNA7 was also ligated into pRSV-CAT plasmid after removal of the chloramphenicol acetyltransferase (CAT) gene. The constructed plasmids were referred to as pEF-mRNA7 and pRSV-mRNA7, respectively (Fig. 1). The CAT gene from pRSV-CAT was ligated into pEF vector (pEF-CAT).

Transfection of the cells: QR32 cells (1×10^6) were cotransfected with 10 μg of pEF-mRNA7 or pRSV-mRNA7, and pSV_2-neo DNA by the standard calcium phosphate precipitation procedure [8]. One day after transfection, cells were trypsinized and transferred to several 60-mm tissue culture dishes and cultured in MEM containing 8% FCS and G418 (1 mg/m*l*). The transfected QR32 clones were obtained by limiting dilution in 96-well culture plates three times. Cloned cells expressing mRNA7 at a high level were selected by Northern blot analysis using cDNA of mRNA7 as a probe. The transfected cell clone expressing mRNA7 of JHMV was referred to as QR-mRNA7. Northern blot analysis was carried out as described previously [18]. An expression of the N protein in QR-mRNA7 cells was confirmed by Western blot analysis. Western blot analysis was carried out as described previously [15].

Mixed lymphocyte cell cultures: C57BL/6 mice were injected in the quadriceps muscle with 100 μg of a closed circular form of plasmid DNA expressing mRNA7 of JHMV. The mice were injected with plasmid DNA once a week at one-week intervals, receiving a total of four injections. The next day after the forth injection, spleens were aseptically removed from the injected mice and homogenized in loose fitting glass homogenizer. The suspensions of spleen cells were then passed through four layers of gauze. The QR-mRNA7 cells to be used as stimulators were treated with mitomycin C (100 μg/m*l*) for 1 hr at 37°C in a 5% CO_2 atmosphere. Spleen cell suspensions (2×10^7) were cultured with the mitomycin C-treated stimulator cells (5×10^5) in 20 m*l* of RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM N-2-hydroxyethylpiperazine-N-2-

ethane sulfonic acid buffer, 50 μM 2-mercaptoethanol, non-essential amino acids and 10% FCS. After 5 days of incubation, the stimulated spleen cells were collected and their cytolytic activity was assessed by ^{51}Cr-release assay.

^{51}Cr-release assay: Target cells (5×10^6) were resuspended in 1 m*l* of medium and labeled with 3.7 MBq sodium [^{51}Cr]chromate (New England Nuclear) for 1 hr. The labeled cells were then washed three times with fresh medium and adjusted to a final concentration of 10^5 cells/m*l*. Aliquots (0.1 m*l*) containing 1×10^4 target cells were distributed into wells in 96-well round-bottom micro-well plates (Corning). Effector cells were added in 0.1 volume of the same medium (effector: target cell ratios of 70:1 to 6:1). Assays were performed in triplicate. After 4 hr incubation, the micro-well plates were centrifuged for 5 min at 1,200 rpm and 0.1 volume of the supernatant was harvested. The radioactivity was measured with a gamma counter, and the specific cytolysis was calculated from the average radioactivity (cpm) of triplicate samples. The percentage of ^{51}Cr-release was assessed by the following formula,

$$\text{Specific } ^{51}\text{Cr-release } (\%) = (a\text{-}c) / (b\text{-}c) \times 100$$

where a is the value of ^{51}Cr-release due to the effector's cytotoxicity, b is the total ^{51}Cr-release caused by treatment with 1 M HCl, and c is the value of spontaneous release from target tumor cells with medium alone. In present study, the spontaneous release was lower than 30% of the total radioactivity incorporated into the target cells.

Chloramphenicol acetyltransferase (CAT) assay: C57BL/6 mice were injected in the quadriceps muscle with 100 μg plasmid DNA expressing CAT gene. Ten days after injection, muscle extracts were prepared from the entire quadriceps according to the method by Wolff *et al.* [28]. Cat assay reactions were performed on 1 μg of total extracts protein for 45 min at 37°C, and products were resolved by thin-layer chromatography according to the method by Gorman *et al.* [7].

RESULTS

Plasmids expressing the CAT gene under the control of Rous sarcoma virus (RSV) LTR or human elongation factor (EF) 1a promoter were injected into the quadriceps muscles of C57BL/6 mice. The CAT activity was observed in the muscle extracts prepared from the mice injected with pRSV-CAT and pEF-CAT plasmid DNA (Fig. 1b). The results showed that the injected plasmid DNAs were incorporated into the muscle cells and that CAT protein was expressed. The specific activity of the muscle extracts from the mice injected with pRSV-CAT was significantly higher than that in those administered pEF-CAT.

We constructed plasmids expressing mRNA7, which codes the N protein of JHMV under the control of RSV LTR or human EF-1a promoter (Fig. 1a). The C57BL/6 mouse fibrosarcoma-derived QR32 cells transfected with these plasmids expressing mRNA7 (QR-mRNA7) were used as stimulator and target cells. When pEF-mRNA7 was injected intramuscularly into C57BL/6 mice and the cytolytic activities of spleen cells were assessed by ^{51}Cr-release assay, only a slight level of cytolysis was observed (Fig. 2). The spleen cells from the mice injected intramuscularly with pRSV-mRNA7 showed high cytolytic activity (9.1%) using QR-mRNA7 cells as a target, even though the cells were not cocultivated with stimulator cells (Fig. 3a). No cytolysis was observed when the untransfected QR32 cells were used as target cells. The spleen cells from the mice receiving pRSV-CAT showed no cytolytic activity against QR32 and QR-mRNA7 cells as a target (data not shown). Therefore, the i.m. injection of pRSV-mRNA7 DNA induced specific cytolytic activity against the cells expressing the viral N protein. When the spleen cells from the mice injected with pRSV-mRNA7 were cocultivated with QR-mRNA7 cells, much higher cytolytic activity (19.6%) was observed using QR-mRNA7 cells as a target (Fig. 3b). However, cytolysis of the untransfected QR32 cells used as target cells was also observed. The levels of cytolysis of QR-mRNA7 cells were significantly higher than those of the untransfected QR32 cells.

DISCUSSION

We constructed plasmids expressing mRNA7, which codes the N protein of JHMV under the control of RSV LTR or human EF-1a promoter (Fig. 1). RSV LTR and human EF-1a promoter direct active expression in various mammalian cells and tissues [6, 11, 20]. Although only a slight level of cytolysis was observed by the spleen cells from C57BL/6 mice injected intramuscularly with pEF-mRNA7, the spleen cells from the mice administered with pRSV-mRNA7 showed a significant level of cytolytic

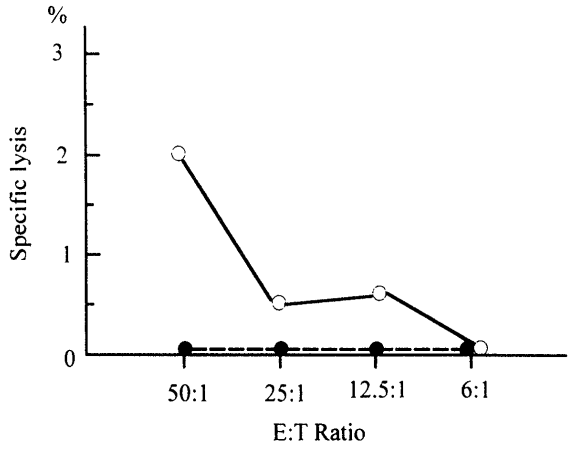

Fig. 2. Cytolytic activities of spleen cells from mice injected with pEF-mRNA7. The spleen cells from C57BL/6 mice injected intramuscularly with pEF-mRNA7 were cocultivated with QR-mRNA7 cells (○) and with QR32 cells (●) used as a stimulator. Specific lysis of QR-mRNA7 cells used as a target was assessed by ^{51}Cr-release assay. Each point represents the average value of 2–3 separate experiments.

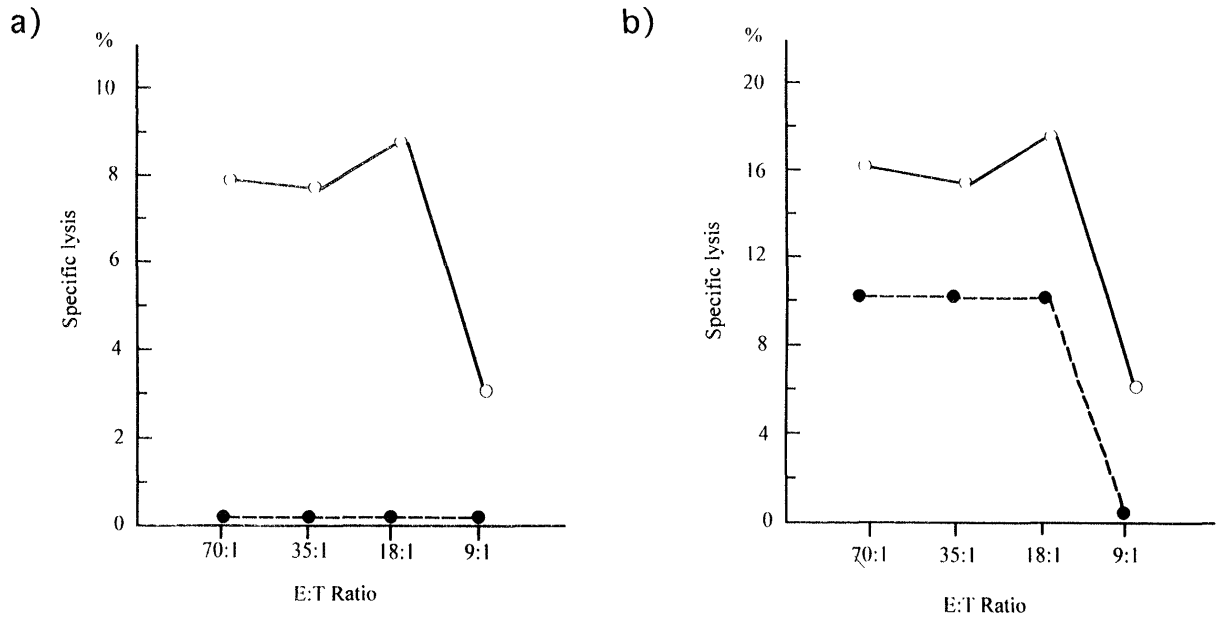

Fig. 3. Cytolytic activities of spleen cells from mice injected with pRSV-mRNA7. pRSV-mRNA was injected intramuscularly into C57BL/6 mice. Specific lysis of control untransfected QR32 cells (●) and QR-mRNA7 cells (○) used as a target by spleen cells which were cultivated without stimulator cells (a) and cultivated with QR-mRNA7 cells (b) was assayed. Each point represents the average value of 2–3 separate experiments.

activities. The difference in the level of specific cytolysis might have been mainly due to the expression levels of the N protein in the muscles between the mice injected with pEF-mRNA7 and pRSV-mRNA7, since the CAT activity in muscles induced by injection with pRSV-CAT plasmid was significantly higher than that with pEF-CAT plasmid. The CAT activity in muscles induced by pRSV-CAT plasmid was much higher than that induced by plasmids expressing CAT gene directed by simian virus 40 early promoter and cytomegalovirus early promoter (data not shown). Therefore, RSV LTR may be an efficient promoter for the expression of genes that is injected into the muscles.

It has been reported that immunization of BALB/c mice (H-2^d) with JHMV induces CTLs that are specific for the N protein [22]. The present results showed that *i.m.* injection of plasmid DNA expressing mRNA7 coding the N protein of JHMV into the muscles of C57BL/6 mice with a different MHC haplotype, H-2^b, from BALB/c mice induced specific cytolytic activity against the cells expressing viral N protein. Although a character of the cells induced by *i.m.* injection of plasmid DNA remains unclear yet, the CTL epitope recognized by C57BL/6 mice (H-2^b) might also be located in the N protein of JHMV. Further investigations concerning a characterization of the induced cells showing cytolytic activity and a response of the QR-mRNA7 cells to spleen cells from mice infected with JHMV are now in progress.

The spleen cells from mice injected intramuscularly with pRSV-mRNA7 showed cytolytic activities against the QR-mRNA7 cells but not against the QR32 cells without cocultivation with stimulator cells. These results suggested that CTLs or the precursor cells of the CTLs specific for the N protein of JHMV may be generated in the spleen of the mouse. When the spleen cells from mice injected with pRSV-mRNA7 were cocultivated with QR-mRNA7 cells as a stimulator, much higher cytolytic activity was observed, compared with the activity without stimulation. The reason why the cytolysis was observed by cocultivated spleen cells against untransfected QR32 cells as a target remains unknown. It has been reported that QR32 cells are resistant to cytolysis caused by natural killer cells but sensitive to that caused by CTLs and lymphokine-activated killer cells (LAK) [19]. Therefore, killer cells nonspecific for antigen such as LAK may be generated.

The generation of nucleoprotein-specific CTLs and protection from a subsequent challenge with a heterologous strain of influenza A virus by *i.m.* injection of plasmid DNA expressing the nucleoprotein of influenza A into BALB/c mice has been reported [25]. Calves injected with a plasmid encoding bovine herpesvirus 1 glycoprotein shed less virus than does the control calf after challenge [4]. Our preliminary result showed that the injection with pRSV-mRNA7 DNA protected against acute infection with JHMV in the brain of the mouse (data not shown). Therefore, injection of DNA that codes the viral proteins may be a potential method of vaccination.

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