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(54) **Title**: RECOMBINANT VACCINIA VIRUS HAVING HEPATITIS C VIRUS GENE

(54) 発明の名称: C 型肝炎ウイルス遺伝子を有する組換えワクシニアウイルス

(57) **Abstract**: Provided is a recombinant virus which is efficacious in preventing the onset of hepatitis C infection and has a high safety. Also provided is a vaccine for hepatitis C virus which contains the recombinant virus. A recombinant vaccinia virus which can express hepatitis C virus gene. The hepatitis C virus vaccine as described above contains the recombinant virus as described above.

(57) 要約: C 型肝炎感染発症の防止に有効性があり安全性の高い組み換えウイルス、およびこれを含む C 型肝炎ウイルス用ワクチンを提供する。本発明にかかる組み換えワクシニアウイルスは、C 型肝炎ウイルス遺伝子を発現することができる。本発明にかかる C 型肝炎ウイルスワクチンは、上記本発明の組み換えウイルスを含むものである。

SPECIFICATION

RECOMBINANT VACCINIA VIRUS HAVING HEPATITIS C VIRUS GENE

FIELD OF THE INVENTION

The present invention relates to a prophylactic agent and a therapeutic agent for hepatitis C. More specifically, the present invention relates to a recombinant vaccinia virus that can express a hepatitis C virus gene, and a prophylactic agent and a therapeutic agent for hepatitis C comprising the recombinant vaccinia virus.

BACKGROUND OF THE INVENTION

There are more than two million people infected with hepatitis C virus (HCV) in Japan, among which about 36,000 people develop hepatocarcinoma every year where most of the cancer patients result in death. Currently, interferon (IFN) is used as the only effective anti-HCV drug, which has limited effect and serious side-effects. Thus, there is a demand for development of a safer and more effective drug. Furthermore, since aging of the infected people increases the risk of developing cancer, there is a need for urgent remedy.

At the current moment, drugs such as nucleic acid analogs, protease inhibitors and the like that suppress viral replication of HCV have been developed and used for treatment. In treatment using these drugs, however, a drug-resistant virus is likely to emerge, and complete elimination of the virus is difficult in view of the mechanisms of this action of the virus, thus a lifelong medication is necessary. In such circumstances, there has been a strong desire for establishment of a curative therapy that allows withdrawal and relief from lifelong medication.

The present inventors have established various experimental model systems, namely, research sources in association with HCV studies by preparing an infectious cDNA clone of HCV and establishing infected animals such as HCV-infectious transgenic mice and human liver chimeric mice and the like (e.g., see Non-patent Document 1). The major features of HCV infection include establishment of persistent infection at a high rate and progress to chronic hepatitis. The present inventors have gone through keen analyses and examinations over the years using the above-mentioned experimental model systems and the like by looking at this mechanism of action in terms of acquisition of immunological tolerance and breakdown thereof (e.g., see Non-patent Document 2).

Numerous attempts to develop a vaccine for preventing HCV infection have been made heretofore but so far none of them provided complete prevention of infection (e.g., see Non-patent Documents 3, 4, 5 and 6).

Non-patent Document 1: Wakita T., et al., J. Biol. Chem., 1998, vol.273, p9001-9006

Non-patent Document 2: Inoue K., et al., Hepatology, 2007, vol.45, p921-928

Non-patent Document 3: Choo QL., et al., Pros. Natl. Acad. Sci. 1994, vol.91, 1294-1298

Non-patent Document 4: Puig M., et. al., Vaccine 2004, vol.22, 991-1000

Non-patent Document 5: Abraham JD., Vaccine 2004, vol.22, 3917-3928

Non-patent Document 6: Elmowalid GA., et. al., Pros. Natl. Acad. Sci. 2007, vol.104, 8427-8432

DISCLOSURE OF THE INVENTION

The present invention provides a therapeutic agent or a prophylactic agent comprising a recombinant vaccinia virus efficacious in preventing the onset of hepatitis C infection.

The present inventors have further gone through keen research based on the results from the above-mentioned analyses and examinations on HCV infection, and came up with an idea that strong immune activation brought about by a recombinant vaccinia vaccine can result in a potent hepatitis C infection prevention method. Moreover, the present inventors also considered that strong activation of the immunological elimination system brought about by a recombinant vaccinia vaccine or the like could provide a potent hepatitis C curative therapy, and thus aiming for complete control of the pathological conditions of a poorly curable viral disease seemed to be possible. The present inventors have also devoted themselves to studies in order to solve the above problem based on their findings from many years of research on viral infections. As a result, they succeeded in preparing a recombinant vaccinia virus that is efficacious in preventing the onset of hepatitis C infection, thereby accomplishing the present invention.

Thus, the present invention is as follows.

(1) A recombinant vaccinia virus comprising an expression promoter and the entire or a part of cDNA of hepatitis C virus genome.

An example of a vaccinia virus includes LC16m8 strain. Examples of cDNAs

of hepatitis C virus genome include those coding for a structural protein of hepatitis C virus or a nonstructural protein of hepatitis C virus, and those coding for both the structural protein of hepatitis C virus and the nonstructural protein of hepatitis C virus.

Specifically, DNAs of (a) to (f) below are exemplified as the cDNAs of hepatitis C virus genome:

(a) DNA having the nucleotide sequence represented by SEQ ID NO:1;

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions, and that codes for a structural protein of hepatitis C virus;

(c) DNA having the nucleotide sequence represented by SEQ ID NO:2;

(d) DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions, and that codes for a nonstructural protein of hepatitis C virus;

(e) DNA having the nucleotide sequence represented by SEQ ID NO:3; and

(f) DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions, and that codes for a structural protein and a nonstructural protein of hepatitis C virus.

Moreover, an example of the expression promoter contained in the recombinant vaccinia virus of the present invention includes a hybrid promoter. Specifically, DNAs of (a) and (b) below are exemplified as a nucleotide sequence of the hybrid promoter:

(a) DNA having the nucleotide sequence represented by SEQ ID NO:4; and

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:4 under stringent conditions and that has a promoter activity.

(2) A pharmaceutical composition comprising the recombinant vaccinia virus according to (1) above.

The pharmaceutical composition may be used as a prophylactic agent or a therapeutic agent for hepatitis C.

The present invention also provides a recombinant vaccinia virus comprising an expression promoter and a cDNA of hepatitis C virus genome, wherein the cDNA is DNA of (a) or (b) below:

(a) DNA consisting of the nucleotide sequence of SEQ ID NO:2; or

(b) DNA that has 90% or more homology with the nucleotide sequence of SEQ ID NO:2, and that codes for a nonstructural protein and a structural protein of hepatitis C

virus.

The present invention also provides a pharmaceutical composition comprising the recombinant vaccinia virus according to the invention.

The present invention also provides use of the recombinant vaccinia virus according to the invention for the manufacture of a medicament for hepatitis C.

The present invention also provides a method for treating hepatitis C in a subject in need of treatment, the method comprising administering the recombinant vaccinia virus according to the invention, or the pharmaceutical composition according to invention, to the subject.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a gene structure of a plasmid used for preparing a HCV recombinant vaccinia virus.

Figure 2 shows the positions and the names of the primers used for confirming HCV gene transfer by PCR.

Figure 3 shows pictures of agarose gel electrophoreses showing the results obtained by confirming HCV gene transfer by PCR.

Figure 4 shows pictures of PVDF membranes showing the results obtained by confirming HCV protein expression by Western blot method.

Figure 5 shows a method for confirming the ability of HCV-RVV to induce humoral/cellular immunity.

Figure 6 shows the results obtained by measuring the effect of HCV-RVV as a vaccine by ELISA method with respect to its ability to induce humoral immunity.

Figure 7 shows the results obtained by measuring the effect of HCV-RVV as a vaccine by ELISPOT assay with respect to its ability to induce cellular immunity.

Figure 8 shows Cre/loxP switching gene expression in a transgenic mouse expressing hepatitis C virus gene.

Figure 9 shows daily changes in the amount of HCV core protein in the liver of the transgenic mouse after the HCV gene expression (Panel A) and tissue alteration in the liver of the transgenic mouse due to onset of hepatitis (Panel B).

Figure 10 shows administration schedule of the HCV recombinant vaccinia virus to the HCV transgenic mice

Figure 11 shows the therapeutic effect after the administration of the HCV recombinant vaccinia virus to the HCV transgenic mice.

BEST MODES FOR CARRYING OUT THE INVENTION

Hereinafter, a recombinant vaccinia virus according to the present invention and an application thereof will be described in more detail although the scope of the present invention should not be limited to these descriptions, and appropriate modification may be performed in a manner apart from the following examples without departing from the scope of the invention.

The present specification incorporates the content of the specifications of Japanese Patent Application Nos. 2008-57515 (filed on March 7, 2008) and 2008-294361 (filed on November 18, 2008), to which the present application claims priority.

Additionally, the patent documents, non-patent documents and other publications cited herein are incorporated herein by reference.

1. Summary

Among various vaccines, live vaccines are some of particularly efficacious vaccines, but development of an attenuated vaccine for a new virus is generally known to

require a very long period of time, which is likely to be the case for HCV as well.

A gene engineering technique for preparing a recombinant vaccinia virus (RVV) as a live vaccine is one of the known techniques employed in such a case. For example, recombinant vaccinia viruses for rabies virus or rinderpest developed by the present inventors have been demonstrated to exert superior effects in preventing the onset of infection in field tests and the like (e.g., see Tsukiyama K., et al., Arch. Virol., 1989, vol.107, p.225-235).

Moreover, the present inventors have succeeded in preparing a recombinant vaccinia virus that has cDNA of SARS-CoV, a known pathogen of atypical pneumonia SARS (WO2006/038742), and confirmed it to be a formulation having a superior prophylactic effect, which can be used for repeated administration (e.g., see Kitabatake M., et al., Vaccine, 2007, vol.25, p.630-637).

A vaccinia virus used as a recombinant parent for preparing RVV needs to be a vaccine strain that has established safety. Vaccinia virus strain LC16m8 (e.g., see Clinical Virology vol.3, No.3, 269, 1975) is known as such a vaccine strain. LC16m8 strain is derived from Lister strain and currently the only vaccine strain that has actually been administrated as a prophylactic vaccine whose safety and efficacy have been confirmed.

The present inventors also found, in the course of developing and studying recombinant vaccinia viruses against rinderpest, HIV, SARS-CoV and the like, that the use of a gene expression promoter that can highly enhance the antibody-producing ability and the cellular immunity-inducing ability is effective for the vaccinia virus of the present invention. Specifically, the present inventors found that pSFJ1-10 or pSFJ2-16 can be used as a preferable plasmid vector (e.g., see Jin N-Y, et al., Arch. Virol. 1994, vol.138, p.315-330, Elmowalid GA., et. al., Pros. Natl. Acad. Sci. 2007, vol.104, 8427-8432; Arch. Virol. 138, 315-330, 1994; Japanese Patent Laid-Open Application No. 6-237773, etc.).

As a result, the present inventors succeeded in preparing a HCV recombinant vaccinia virus by integrating a gene coding for a nonstructural protein of HCV and/or a gene coding for a structural protein of HCV together with a promoter into a vaccinia virus.

A parental virus of a recombinant vaccinia virus of the present invention is a vaccinia virus as described above. A recombinant vaccinia virus of the present invention has cDNA of HCV integrated into the genome of the vaccinia virus. An expression unit obtained by cloning the entire gene regions encoding the HCV protein, the outer capside protein region, or the gene of the nonstructural protein region associated

with replication is transferred into a vaccinia virus vector. This expression unit is introduced into the HA-coding region of the vaccinia virus. Since foreign gene transfer into the HA-coding region has no influence on the proliferation activity of the vaccinia virus, as is already known, a safe vaccine strain having weak proliferating ability can be used as the parental virus (Vaccine 12, 675-681, 1994).

Live recombinant vaccinia vaccines against rabies virus, rinderpest virus and the like have been field-tested, where their excellent prophylactic effects have been proved against the onset of respective infections.

The present inventors prepared a recombinant vaccinia virus (RVV) by inserting the entire gene coding for hepatitis C virus (HCV) protein, a gene coding for the outer capside protein region, or a gene coding for a replication-associated nonstructural protein region downstream from a hybrid promoter, and integrating these genes into the hemagglutinin (HA) gene region of an attenuated vaccinia virus strain.

The hybrid promoter includes a poxvirus A-type inclusion (ATI) promoter and a vaccinia virus 7.5 kDa protein (p7.5) early expression promoter with multiple repeats (see Jin N-Y, et al., Arch. Virol. 1994, vol.138, p.315-330). This promoter was developed by and available from Dr. Hisatoshi Shida at Hokkaido University.

The prepared RVV was used to infect an animal cell, by which abundant expression of HCV protein as well as earlier production of a high-titer antibody against HCV were confirmed by vaccination to an animal individual. Additionally, cellular immunity was also confirmed to be activated upon vaccination to an animal individual by ELISPOT assay, thereby accomplishing the present invention.

2. Preparation of HCV recombinant vaccinia virus

The entire gene coding for hepatitis C virus (HCV) protein, a gene coding for the outer capside protein region, and a gene coding for a replication-associated nonstructural protein region have already been cloned and inserted into a plasmid. Hence, a gene contained in the recombinant virus of the present invention can be obtained according to a usual gene engineering technique. For example, a nucleic acid synthesis method using a generally-used DNA synthesizer may be employed as such a gene engineering technique. Moreover, after isolating or synthesizing a gene sequence as a template, a PCR method may be employed in which primers specific to each gene are designed to amplify the gene sequences with a PCR device or a gene amplification method using a cloning vector. The above-mentioned methods may readily be carried out by those skilled in the art according to Molecular cloning 2nd Ed. Cold Spring Harbor Laboratory Press (1989) or

the like. The obtained PCR product may be purified according to a known method.

In a preferred embodiment of the present invention, the HCV gene inserted into the above-described plasmid (Genotype 1b; Nucleotide Number: 1-9611; DDBJ/EMBL/GenBank accession number: AY045702) may be used as a template. Thus, HCV gene cDNA is used as a template with HCV gene-specific primers to perform PCR, thereby preparing each gene region of HCV. According to the present invention, the entire gene regions of HCV, a gene coding for the structural protein region of the outer capside protein and a gene coding for the replication-associated nonstructural protein region are referred to as "CN5", "CN2" and "N25", respectively.

The nucleotide sequences of CN2, N25 and CN5 are represented by SEQ ID NOS:1, 2 and 3, respectively. Other than the DNAs of the sequences represented by SEQ ID NOS:1 to 3, the following DNAs may also be used for the present invention.

DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:1 under stringent conditions, and that codes for a structural protein of hepatitis C virus (mutant DNA of CN2);

DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:2 under stringent conditions, and that codes for a nonstructural protein of hepatitis C virus (mutant DNA of N25);

DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:3 under stringent conditions, and that codes for a structural protein and a nonstructural protein of hepatitis C virus (mutant DNA of CN5).

Herein, the phrase "coding for a structural protein of hepatitis C virus" means that the gene codes for a protein constituting the outer capside of the virus, specifically, the gene codes for at least the core region, E1 region and E2 region (Figure 1A). Furthermore, the phrase "coding for a nonstructural protein of hepatitis C virus" means that the gene codes for a protein produced in the cell upon propagation of the virus, specifically, the gene codes for at least NS2 region, NS3 region, NS4a region, NS4b region, NS5a region and NS5b region (Figure 1B).

In addition, the above-described genes coding for the structural protein and the nonstructural protein comprise the full-length sequence as well as a partial sequence thereof. In the case of CN2, it may not necessarily be a full-length sequence and may be a part thereof as long as it contains all or any of the core region, E1 region and E2 region. For example, E1 region (589-1164) and E2 region (1165-2253) of the nucleotide sequence represented by SEQ ID NO:1 may be used. In the case of N25, it may not

necessarily be a full-length sequence and may be a part there of as long as it contains all or any of NS2 region, NS3 region, NS4a region, NS4b region, NS5a region and NS5b region. For example, NS2 region (805-1455) and NS3 region (1456-3348) of the nucleotide sequence represented by SEQ ID NO:2 may be used. In the case of CN5, it may not necessarily be a full-length sequence and may be a part thereof as long as it contains all or any of the core region, E1 region, E2 region, NS2 region, NS3 region, NS4a region, NS4b region, NS5a region and NS5b region. For example, E1 region (589-1164), E2 region (1165-2253), NS2 region (2443-3093) and NS3 region (3094-4986) of the nucleotide sequence represented by SEQ ID NO:3 may be used.

The above-described mutant DNA may be obtained by chemical synthesis, or it may alternatively be obtained from a cDNA library or a genome library by a known hybridization method such as colony hybridization, plaque hybridization, Southern blot or the like using DNA having the nucleotide sequence represented by any of SEQ ID NOS:1-3 or a fragment thereof as a probe. Examples of stringent conditions for the above-mentioned hybridization include 0.1xSSC to 10xSSC, 0.1% to 1.0% SDS and 20°C to 80°C. More specifically, after performing prehybridization at 37°C to 56°C for 30 minutes or longer, washing is carried out for 1 to 3 times in 0.1xSSC, 0.1% SDS at room temperature for 10 to 20 minutes. For specific procedure of the hybridization method, reference may be made to "Molecular Cloning, A Laboratory Manual 2nd ed." (Cold Spring Harbor Press (1989)) or the like.

Moreover, DNA (mutant DNA of CN2) that has 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, 95% or more, 98% or more or 99% or more homology with the nucleotide sequence represented by SEQ ID NO:1 and that codes for the structural protein of hepatitis C virus, DNA (mutant DNA of N25) that has 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, 95% or more, 98% or more or 99% or more homology with the nucleotide sequence represented by SEQ ID NO:2 and that codes for the nonstructural protein of hepatitis C virus, and DNA (mutant DNA of CN5) that has 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, 95% or more, 98% or more or 99% or more homology with the nucleotide sequence represented by SEQ ID NO:3 and that codes for the nonstructural protein and structural protein of hepatitis C virus may be used.

A promoter contained in the recombinant vaccinia virus of the present invention is a hybrid promoter consisting of a poxvirus A-type inclusion (ATI) promoter and a vaccinia virus 7.5 kDa protein (p7.5) early expression promoter with multiple repeats included in the hemagglutinin (HA) gene region of the vaccinia virus. This promoter

may be linked to an appropriate plasmid, for example, pBMSF7C (Arch. Virol. 138, 315-330, 1994; Japanese Patent Laid-Open Application No. 6-237773).

A nucleotide sequence of a hybrid promoter that can be used for the present invention is represented by SEQ ID NO:4. Besides DNA having the nucleotide sequence represented by SEQ ID NO:4, DNA that hybridizes with DNA having a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO:4 under stringent conditions and that has a promoter activity may also be used for the present invention. The "stringent conditions" are the same as describe above. The phrase "having a promoter activity" means to have an activity for transcripting a gene coding for a structural protein or a nonstructural protein.

A protein may be mass-expressed by this hybrid promoter in a completely sugar-modified form through early to late vaccinia virus infection. According to the present invention, a plasmid vector having HCV gene (CN5) inserted downstream from a pBMSF7C promoter is referred to as pBMSF7C-CN5. Moreover, according to the present invention, a plasmid vector having an outer capside protein region gene (CN2) inserted downstream from a pBMSF7C promoter is referred to as pBMSF7C-CN2. Furthermore, a plasmid vector having a nonstructural protein gene (N25) inserted downstream from pBMSF7C promoter is referred to as pBMSF7C-N25.

These plasmid vectors are transferred into a vaccinia virus as a host to prepare a recombinant vaccinia virus. For transfer into the host plasmid vector, any known technique may be employed. For example, any one of plasmid vectors pBMSF7C-CN5, pBMSF7C-CN2 and pBMSF7C-N25 can be introduced into an animal cell infected with an attenuated vaccinia virus strain LC16m8 to induce homologous recombination in the hemagglutinin (HA) gene region of the vaccinia virus to prepare recombinant vaccinia viruses (RVV-CN5, RVV-CN2 and RVV-N25) expressing the respective HCV proteins.

The vaccinia virus strain LC16m8 used for preparing RVV is an attenuated strain that may proliferate in an animal individual but has extremely low proliferating property in the nerve cells. This strain is approved as a smallpox vaccine in Japan and no serious side-effect has occurred from vaccination to approximately 50,000 children (research report by the smallpox vaccination research group at the Ministry of Health and Welfare, Clinical Virology vol.3, No.3, 269, 1975). On the other hand, its immunity-inducing ability is reported to be equivalent to that of Lister strain, i.e., the parent strain, and thus LC16m8 strain is a safe and effective vaccine.

Since the prepared RVV-CN5, RVV-CN2 and RVV-N25 have the HCV protein gene inserted into the HA gene region of the vaccinia virus, expression of HA protein is

defected and thus no hemagglutination is caused. Accordingly, RVV-CN5, RVV-CN2 and RVV-N25 are used to infect animal cells, and RVV is screened using agglutination reaction of chicken erythrocyte with the resulting plaque as the indicator. The RVV of interest may be obtained by selecting white plaques in which no hemagglutination is observed.

Gene transfer of HCV in the virus obtained from the white plaques may be confirmed by performing PCR using the virus genome as a template and HCV gene-specific primers.

Expression of HCV protein may be confirmed by Western blot method using animal cells infected with RVV-CN5, RVV-CN2 and RVV-N25 as samples. Here, the antibody used may be obtained by purifying IgG with Protein G from an antiserum raised by immunization with an HCV polypeptide (J. Biol.Chem. 279:14531-14541, 2004).

Other than the HA gene region, thymidine kinase (TK) gene region is generally used as the insertion site for the gene of interest upon preparation of RVV but insertion of the gene of interest into the TK gene region is known to lower the RVV proliferation due to the defect in TK expression. On the other hand, the defect in HA protein expression is reported to have little effect on the RVV proliferation (Vaccine 12, 675-681, 1994). Therefore, according to the present invention, the insertion site of the gene of interest is preferably the HA gene region.

3. Pharmaceutical composition for preventing or treating hepatitis C

The present invention provides a prophylactic and therapeutic agent (a pharmaceutical composition) for hepatitis C comprising the above-described recombinant vaccinia virus.

A pharmaceutical composition of the present invention may be introduced into an organism by any known method such as intramuscular, intraperitoneal, intradermal or subcutaneous injection; nasal, buccal or lung inhalation; or oral administration. Additionally, a recombinant virus contained in a pharmaceutical composition of the present invention may be used in combination with an existing antiviral drug (for example, interferon). An embodiment of combinational use is not particularly limited, and the recombinant virus of the present invention and the existing antiviral drug may be introduced into an organism by a method in which both are administered simultaneously or by a method in which one is administered after the other at a certain interval.

Moreover, the pharmaceutical composition of the present invention may be blended with a known pharmaceutically acceptable carrier such as an excipient, a bulking

agent, a binder and a lubricant, a buffer, a tonicity agent, a chelating agent, a colorant, a preservative, a fragrance, a flavoring agent, a sweetening agent or the like.

The pharmaceutical composition of the present invention may orally or parenterally be administered according to its form, for example, as an orally administered agent such as a tablet, a capsule, a powdered agent, a granular agent, a pill, a solution, syrup or the like, or a parenterally administered agent such as an injection, a topical agent, a suppository, an eye drop or the like. Preferably, it is, for example, a local injection such as an intradermal, intramuscular or intraperitoneal injection.

Although the dosage is appropriately chosen according to the type of the active element, administration route, administration target, age, weight, sex and symptoms of the patient and other conditions, the daily dosage of the virus is about 1,000-1,000,000,000 PFU (plaque forming units) and preferably about 100,000-100,000,000 PFU in the case of oral administration, while it is about 100-1,000,000,000 PFU and preferably about 1,000-100,000,000 PFU in the case of parenteral administration. The virus may be administered once or several times a day.

The recombinant virus of the present invention may be used as a vaccine for preventing or treating hepatitis C. Furthermore, development of a vaccine against HCV to date is based on the research focusing on antibodies against HCV and cytotoxic T cells (CTL). Therefore, the antibody titer or the cellular immunity activity as a vaccine is preferably measured beforehand.

For example, the antibody titers against the prepared RVV-CN5, RVV-CN2, RVV-N25 or LC16m8 strain, i.e., the parent strain, may be obtained by vaccinating a rabbit with the virus strain, collecting the sera over time, and measuring the ELISA value against HCV in the sera. In the sera of the rabbit vaccinated with RVV-CN5- or RVV-CN2, antibody titers against HCV increased after four weeks following the vaccination.

In addition, the cellular immunity activity can be measured by vaccinating a mouse with RVV-CN5, RVV-CN2, RVV-N25 or LC16m8 strain, i.e., the parent strain, isolating the spleen cells from the immunized mouse, and determining whether or not HCV-specific CTL is induced by ELISPOT assay. According to the present invention, when the spleen cells derived from RVV-CN5-vaccinated BALB/c mouse were stimulated with a synthetic peptide of a H-2^d-restricted E1-specific CTL epitope sequence, INF-γ-producing cells of nearly ten times the control were detected. Accordingly, RVV-CN5 vaccination was found to induce E1-specific CTL in BALB/c mice.

Thus, HCV-RVVs prepared by the present inventors have been confirmed to

induce humoral and cellular immunity against HCV.

The present invention will be described more specifically by the following examples. These examples are for illustration only and should not limit the scope of the present invention.

[EXAMPLE 1]

Preparation of recombinant vaccinia virus

The entire gene regions (CN5), the outer capside protein region (CN2) and the gene region of the replication-associated nonstructural protein region (N25) of hepatitis C virus (DDBJ/EMBL/GenBank accession number; AY045702) were integrated into SbfI and SgfI Sites of pBMSF7C plasmid (Japanese Patent Laid-Open Application No. 6-237773) to prepare plasmid vectors pBMSF7C-CN5, pBMSF7C-CN2 and pBMSF7C-N25 having the HCV genes inserted downstream from the ATI·p7.5 hybrid promoter in hemagglutinin (HA) gene region (Figure 1).

Primary cultured kidney cells were seeded into a T175 flask. Once the cells reached to confluence, the attenuated vaccinia virus strain LC16m8 was used for infection at moi=10 and at 30°C for 2 hours. Here, moi (multiplicity of infection) refers to PFU per cell. Following infection, the virus solution was removed by suction, and the cells were washed with PBS(-). Then, after treatment with 0.05% trypsin/0.5mM EDTA/PBS(-) and washing with 10% FCS/MEM medium, PBS(-) and HeBS buffer, the cells were suspended in 600 μl HeBS buffer. 40 μg each of plasmid vectors pBMSF7C-CN5, pBMSF7C-CN2 and pBMSF7C-N25 was diluted using a HeBS buffer to obtain a total amount of 200 μl, which was added to and mixed with the cell suspension and left to stand on ice for 10 minutes. The cell suspension added with the plasmid vector was transferred to a 0.4 cm cuvette to perform electroporation (0.2 kV, 960 μF) using an electroporator (Bio-Rad). After electroporation, 1 ml of 10% FCS/MEM medium was immediately added to the cell suspension for dilution. This cell suspension was added to RK13 cell or primary cultured kidney cell that had been seeded into a T175 flask and cultured at 30°C for 24 hours.

After 24 hours of cultivation, the culture supernatant was removed by suction and the cells were washed with PBS(-). Next, treatment with 0.05% trypsin/0.5mM EDTA/PBS(-) was performed and then the cells were suspended in a 10%FCS/MEM medium. The cell suspension was collected, subjected to ultrasonication (30 sec x 4 times) in cold water and then centrifuged (2000 rpm, 10 min). The resulting supernatant was used as a virus solution. The virus solution was diluted in 10% FCS/MEM medium,

and used to infect the primary cultured kidney cell that had been seeded onto a 100 mm dish at 30°C for an hour. The virus solution was removed by suction, and then the cells were washed with PBS(-). 10% FCS/0.5% methylcellulose/MEM medium was added for cultivation at 30°C for 72 hours. After 72 hours of cultivation, the supernatant was removed by suction and washed with PBS(-). A chicken erythrocyte solution diluted in PBS(+) was added to the 100 mm dish for cultivation at 37°C for 30 minutes. The erythrocyte solution was removed by suction and then the cells were washed twice with PBS(-). Plaques onto which chicken erythrocyte was unadsorbed were collected using a pipetman. HCV gene transfer in the collected plaques was confirmed by PCR and gene sequencing. Plaques confirmed of gene transfer were subjected to plaque purification for three times.

The viruses subjected to three times of plaque purification were subjected to small-scale cultivation. The colony obtained after the third purification was suspended in 700 μl of 10% FCS/MEM medium and subjected to ultrasonication in cold water. Following centrifugation (2000 rpm, 10 min), 500 μl of supernatant was added to primary cultured kidney cells seeded in T25 for infection at 30°C for 2 hours. After the infection, the virus solution was removed by suction and the cells were washed with 2.5 ml of 10% FCS/MEM medium. The medium was removed by suction and 2.5 ml of 10% FCS/MEM medium was newly added for cultivation at 30°C for 72 hours. After 72 hours, the cells were scraped off from the flask with a scraper to collect the cell suspension. The collected cell suspension was subjected to ultrasonication (30 sec, 4 times) in cold water, followed by centrifugation, and the supernatant was collected as the virus solution. The collected virus solution was serially diluted and then added to RK13 cells or primary cultured kidney cells that had been seeded onto 6-well plates for infection at 30°C for an hour. The virus solution was removed by suction and the cells were washed twice with PBS(-) and added with 10% FCS/0.5% methylcellulose/MEM medium for cultivation at 30°C for 72 hours. After 72 hours, the number of plaques formed in the well was counted to calculate the titer.

Based on this calculated titer, mass-scale culture was performed. RK13 cells or primary cultured kidney cells were seeded into ten T175 flasks. Once the cells reached confluence, the recombinant vaccinia virus solution was used for infection at moi=0.1 and at 30°C for 2 hours. Following infection, the virus solution was removed by suction and the cells were washed with 20 ml of 10% FCS/MEM medium. The medium was removed by suction and 20 ml of 10% FCS/MEM medium was newly added to culture at 30°C for 72 hours. After 72 hours, the cells were scraped off from the flasks using a

scraper, and the cell suspensions were collected and frozen at -80°C for preservation. This cell suspension was subjected to three rounds of freezing and thawing, followed by ultrasonication (30 seconds, 4 times) in cold water and centrifugation to collect the supernatant as a virus solution. The collected virus solution was transferred to a high-speed centrifugation tube and subjected to centrifugation at 18000 rpm for 45 minutes to allow precipitation of the virus. The supernatant was removed by suction, and then the pellets were resuspended in a small amount of 10% FCS/MEM medium. By this procedure, a virus solution that was concentrated ten times stronger than that with the T175 flask cultivation was prepared. This concentrated virus solution was serially diluted and used to infect RK13 cells or primary cultured kidney cells that had been seeded onto a 6-well plate, and the virus titers of the solutions were calculated in the same manner as the above-described method. The concentrated virus solutions with calculated titers were used in various experiments described in the following examples.

[EXAMPLE 2]

Confirmation of HCV gene transfer by PCR

PCR was performed using the following primers specific to HCV gene and the obtained recombinant vaccinia virus genome as a template to confirm whether or not HCV gene was introduced into the virus genome (Figures 2 and 3).

(1) Nucleotide sequences of primers for cloning

Fw: HCV-CL-Fw (SEQ ID NO:5)

5-GGGCGGCCCTGCAGGTAATACGACTCACTATAGGGCGTAGACCGTGCATCATGAGCACAAATCCTAAACCCCAAAGAAAAACCAAACG-3

Rv: HCV-CL-MRv (SEQ ID NO:6)

5-GGGCGGCGCGATCGCCTATCATTAAAGGAGCCGCCACCCCTGCCCTTCAAGACTATC-3

Fw: HCV-CL-MFw (SEQ ID NO:7)

5-GGGCGGCCCTGCAGGTAATACGACTCACTATAGGGCGTAGACCGTGCATCATGACGCGGCCGCCGCAAGGCAACTGGTTCGGC-3

Rv: HCV-CL-Rv (SEQ ID NO:8)

5-GGGCGGCGCGATCGCCTATCATTATCGGTTGGGGAGCAGGTAGAT

GCCTAC-3

(2) Nucleotide sequences of primers for PCR for confirming the insert

<HA>

Fw: HA-1-S (SEQ ID NO:9)

5-GGTCTTATATACACCGAGTAAGG-3

Rv: PBSF-110-350-R20 (SEQ ID NO:10)

5-TCAGGAAAGACAGCCATAGC-3

<First half region>

Fw: PBSF-110-1204-S22 (SEQ ID NO:11)

5-CATCACATTGAAACATTGGGAC-3

Rv: 6-354-R20 (SEQ ID NO:12)

5-GATTTGTGCTCATGATGCAC-3

Rv: 6-2139-R23 (SEQ ID NO:13)

5-CCGAACCACATTTTGTGTAAGTG-3

<Latter half region>

Fw: 6-3251-18S (SEQ ID NO:14)

5-AGTAGAGCCCGTTGTCTT-3

Fw: 6-9168-S20 (SEQ ID NO:15)

5-TACCTCTTCAACTGGGCAGT-3

Rv: HA-6-R (SEQ ID NO:16)

5-CTAGTTCTGAGAAACCAGAGG-3

Specifically, the composition of the reaction solution was 1U DNA polymerase, 0.3 mM dNTP, 1 μM F primer and 1 μM R primer in 50 μL of buffer that comes with a commercially available polymerase. The cycle conditions were 25 cycles of: denaturing at 95°C for 0.5 minutes; annealing at 58°C for 0.5 minutes; and elongation at 72°C for 2 minutes. The resulting PCR product was subjected to electrophoresis using an agarose

gel to confirm the band. As a result, if a single band having the length anticipated based on the primer design was observed, HCV gene was considered to be transferred into the recombinant virus genome whereas HCV gene was not transferred if no such band was observed.

As shown in Figure 3, as a result of 2 wt% agarose gel electrophoresis of the PCR product (amplified fragment), HCV gene was found to be transferred into the recombinant virus genome.

[EXAMPLE 3]

Confirmation of HCV protein expression by Western blot method

The recombinant vaccinia virus RVV-S was used to infect RK13 cells that had been seeded onto a 6-well plate at moi=30 and at 30°C for 2 hours. After infection, the virus solution was removed by suction and the cells were washed twice with PBS(-). To each well, 2 ml of 10% FCS/MEM medium was added for cultivation at 30°C for 24 hours. After 24 hours, the medium was removed by suction, and 100 μl of lysis buffer (1% SDS, 0.5% NP-40, 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4)) was added to lyse the cells, and the resultant solution was transferred into a 1.5 ml Eppendorf tube. The collected solution was subjected to ultrasonication in cold water until viscosity became zero. The protein amount in the prepared solution was quantified according to Lowry method.

Electrophoresis was carried out for 50 μg of protein with 10% acrylamide gel. At the end of the electrophoresis, the gel was removed, and the protein in the gel was transferred onto a PVDF membrane with a semi-dry blotter by running a current at 5.5 mA/cm^2 for 60 minutes. After washing the membrane with a TBS-T solution, the membrane was immersed into a 5% skimmed milk-TBS-T solution for blocking. After blocking, the membrane was washed for three times with a TBS-T solution. The primary antibody was a mouse monoclonal antibody obtained by purifying Core-31-2, E1-384, E2-544, NS3-10-1, NS4B-52-1 and NS5B-14-5 clone IgG. The protein amount of the purified antibody was quantified by Lowry method and prepared to be 10 μg/ml for use. At the end of the reaction with the primary antibody, the membrane was washed for three times with a TBS-T solution. The secondary antibody used was anti-rabbit IgG-linked HRPO (from Donkey, Amersham). At the end for the reaction with the secondary antibody, the membrane was again washed for three times with a TBS-T solution, and an ECL solution was added to the membrane for film development.

Consequently, as shown in Figure 4, HCV protein was found to be expressed in

the recombinant virus genomes RVV-CN2, RVV-N25 and RVV-CN5.

[EXAMPLE 4]

Experiments of vaccination of rabbits and mice with recombinant vaccinia viruses (Figure 5)

Figure 5 shows a method for confirming the ability of HCV-RVVs to induce humoral/cellular immunity.

New Zealand white female rabbits were transendothelially vaccinated with the recombinant vaccinia viruses or the parent strain LC16m8 obtained in Example 1 at $1x10^8$ pfu. Blood was drawn from the ear vein after 1, 2, 3, 4 and 6 weeks following the vaccination. Furthermore, after six weeks following the initial viral vaccination, RVV-S was used again for vaccination at $1x10^8$ pfu. Similarly, blood was again drawn from the ear vein after 1, 2, 3, 4 and 6 weeks following the second vaccination. All of the collected blood was drawn into vacuum blood-collecting tubes (TERUMO, trade name: Venoject II vacuum blood-collecting tubes (sterile), 9 mL), and subjected to centrifugation (3000rpm, 20 minutes) to separate and collect the sera. The sera were frozen at -20°C for preservation until the later-described ELISA test.

[EXAMPLE 5]

Measurement of antibody titers against HCV protein in sera of HCV-RVV-vaccinated rabbits by ELISA method

A 96-well plate was coated with core protein and E2 protein, to which a 100-fold dilution of the frozen serum sample was added. After leaving it to stand at room temperature for an hour, the 96-well plate was washed with a TBS-T solution and then anti-rabbit IgG-linked HRPO (from Donkey, Amersham) was added to the 96-well plate as a secondary antibody. After the reaction with the secondary antibody at room temperature for an hour, the 96-well plate was again washed for three times with a TBS-T solution, to which a color development solution was added at 100 μl/well. After leaving it to stand at room temperature for 10-20 minutes, absorbance at 450 nm was measured with a microplate reader.

As a result, as shown in Figure 6, higher antibody titers were induced against core and E2 in the sera of RVV-CN2- and RVV-CN5-vaccinated rabbits.

[EXAMPLE 6]

Confirmation of cellular immunity-inducing abilities of HCV-RVVs as vaccines

by ELISPOT assay

(Day 1)

The purified anti-mouse IFN-γ antibody (R4-6A2) (1μg/ml) (Pharmingen) was seeded onto a 96-well nitrocellulose plate at 75 to 100 μl/well while adjusting the final concentration to be 8 μg/ml (125-fold diluted in sterile PBS) and left to stand at 4°C overnight.

(Day 2)

Spleen cells were collected from the mouse and allowed to suspend at a suitable amount in washing RPMI. The washing RPMI used was supplemented with 2.5% FCS. The cells were collected by centrifugation at 1200 rpm at 4°C for 5 minutes. The cells were treated with ACK, suspended in washing RPMI at a suitable amount, and again centrifuged at 1200 rpm and at 4°C for 5 minutes to collect the cells. 500 μl of washing RPMI followed by the cell suspension were forced to pass through a filter. After complete passage, the cells were washed with 1.5 ml of washing RPMI. The resultant was washed once with 10% FCS-supplemented RPMI, suspended in H-h medium and adjusted to $1x10^7$/ml.

1) H-h medium: A mixture of equal amounts of 10% FCS-supplemented RPMI and 10% FCS-supplemented CLICK'S medium.

2) 10% FCS-supplemented RPMI: RPMI-1640 (SIGMA R8758), FCS (final 10%), 2-mercaptoethanol (final concentration 5μM), penicillin-streptomycin (final concentration PCs:100 u/ml, SM:0.1 mg/ml) and 7.5% $NaHCO_3$ 4ml

3) 10% FCS-supplemented CLICK'S medium: CLICK'S medium (SIGMA C5572), FCS (final concentration 10%), 2-mercaptoethanol (final concentration 5μM), penicillin-streptomycin (final concentration PCs:100 u/ml, SM:0.1 mg/ml) and 7.5% $NaHCO_3$ 4ml

Initiation of cultivation

The 96-well nitrocellulose plate was washed for three times with PBS (100 μl/well), added with 10% FCS-supplemented RPMI at 100 μl/well, and placed in a CO_2 incubator at 37°C for an hour for blocking. The medium was discarded, and effector cells were seeded in two-fold serial dilution from $1x10^6$/100 μl/well to $0.125x10^6$/100 μl/well.

A peptide solution (200 μg/ml) was added at 100 μl/well (final concentration

100 μg/ml) for cultivation in a CO_2 incubator at 37°C for 24 hours.

(Day 3)

The medium was discarded, and the resultant was washed for ten times with PBS, 0.05% Tween 20 (200 μl/well). The biotinated anti-mouse IFN-γ (XMG1.2) (0.5 mg/ml) (Pharmingen) was adjusted to a final concentration of 2 μg/ml (250-fold diluted in PBS) and added at 100 μl/well. The resultant was left to stand at 4°C overnight.

(Day 4)

The 96-well nitrocellulose plate was washed for ten times with PBS, 0.05% Tween 20 (200 μl/well). Streptavidin-alkaline phosphatase (1 mg/ml) (MABTECH AB) was adjusted to a final concentration of 1 μg/ml (1000-fold diluted in PBS) and added at 100 μl/well.

The resulting solution was left to stand at room temperature for 1.5 hours. A 25x AP color development buffer (BIO-RAD) was 25-fold diluted with DW and 1/100 amounts of color reagents A and B (BIO-RAD) were added to prepare a reaction mixture. The 96-well nitrocellulose plate was washed for ten times with PBS, 0.05% Tween 20 (200 μl/well). The reaction mixture was added at 100 μl/well and left to stand at room temperature for 10-20 minutes. Once color was developed and a dark spot appeared, the reaction mixture was discarded and thoroughly washed in water. The bottom of the 96-well nitrocellulose plate was peeled off and dried to count the number of spots with ELISPOT Reader.

The resulting counts are shown in Figure 7, where strong cellular immunity was induced.

[EXAMPLE 7]

Examination of therapeutic effect of HCV-RVVs against hepatitis C

A transgenic (Cre/loxP/HCV-Tg) mouse (in Figure 8, "loxP-HCV") transferred with HCV gene with a Cre/loxP system was mated with an IFN-induced Cre-expressing transgenic mouse (in Figure 8, "MxCre") to prepare a Tg mouse (Cre/loxP/HCV-MxCre Tg) that switchingly expresses HCV gene at an arbitrary time (Figure 8) for analyzing the pathological condition thereof. For analysis of the pathological condition, poly IC, i.e., an interferon-inducing agent, was used in order to allow gene expression of recombinant enzyme Cre with interferon.

Here, in Figure 8, Panel A shows expression of HCV gene by Cre/loxP

switching system while Panel B shows mating between the loxP-HCV transgenic mouse and the MxCre gene-expressing transgenic mouse.

As HCV-RVVs, RVV-CN2 that predominantly expresses the structural protein of HCV, RVV-N25 that expresses the replication-associated nonstructural protein, and RVV-CN5 that expresses the whole protein were used (Figure 1). In order to assess the therapeutic effects of these HCV-RVVs, Cre/loxP/HCV-MxCre Tg mice that persistently expressed HCV protein for 3 months were intradermally vaccinated once with the HCV-RVVs (1×10^7 pfu), and the mouse livers were sampled after four weeks following the vaccination (Figure 9). Subsequently, expression levels and morphology of HCV proteins in the mouse livers were examined.

The results are shown in Figure 10. In Figure 10, Panel A shows the transitions of HCV core protein amount ("HCV core") and ALT (alanine aminotransferase), in which the HCV core protein amount ("HCV core") and ALT are represented by a bar graph and a line graph, respectively. ALT is an indicator of the degree of liver damage. In Panel B, "d0", "d90", "d180" and "d480" indicate tissue alteration in the liver before the HCV gene expression, and after 90 days, 180 days and 480 days following the HCV gene expression, respectively.

The HCV protein in the liver of the Cre/loxP/HCV-MxCre Tg mouse (in Figure 10, "HCV core") was not completely eliminated and persistent expression was confirmed for more than a year, indicating a pathological condition of chronic hepatitis such as inflammation or adipose degeneration, fibrosis and the like in the liver (Figure 10).

Furthermore, in the livers of the Cre/loxP/HCV-MxCre Tg mice after four weeks following the HCV-RVV vaccination, the expression level of the core protein was decreased for the RVV-N25 vaccination group (Figure 11). Moreover, for the RVV-N25 group, morphological abnormality in the liver (cord-like structure of the liver, conditions of the liver cells or the like) returned to normal (Figure 11).

In Figure 11, "m8", "CN2", "CN5" and "N25" represent LC16m8 strain, RVV-CN2, RVV-CN5 and RVV-N25, respectively. Panel A shows the amounts of HCV core proteins in the livers while Panel B shows images of the liver tissues after four weeks following the HCV-RVV vaccination. In Panel B, "a" shows the image of the liver tissue prior to the HCV gene expression while "b" shows the images of the liver tissues after four weeks following the HCV-RVV vaccination. As can be appreciated from Figure 11, the liver of the mouse administered with RVV-N25 returned to normal. Thus, the vaccinia virus of the present invention was shown to have a therapeutic effect on hepatitis C.

INDUSTRIAL APPLICABILITY

According to the present invention, there are provided a novel recombinant vaccinia virus that is efficacious and highly safe in preventing or treating hepatitis C, and a prophylactic or therapeutic agent for hepatitis C (a vaccine for preventing or treating hepatitis C) comprising the novel virus.

[Sequence Listing]

SEQ ID NO:5: Synthetic DNA

SEQ ID NO:6: Synthetic DNA

SEQ ID NO:7: Synthetic DNA

SEQ ID NO:8: Synthetic DNA

SEQ ID NO:9: Synthetic DNA

SEQ ID NO:10: Synthetic DNA

SEQ ID NO:11: Synthetic DNA

SEQ ID NO:12: Synthetic DNA

SEQ ID NO:13: Synthetic DNA

SEQ ID NO:14: Synthetic DNA

SEQ ID NO:15: Synthetic DNA

SEQ ID NO:16: Synthetic DNA

SEQ ID NO:17: Synthetic peptide

SEQ ID NO:18: Synthetic peptide

SEQ ID NO:19: Synthetic peptide

SEQ ID NO:20: Synthetic peptide

Claims

1. A recombinant vaccinia virus comprising an expression promoter and a cDNA of hepatitis C virus genome, wherein the cDNA is DNA of (a) or (b) below:

(a) DNA consisting of the nucleotide sequence of SEQ ID NO:2; or

(b) DNA that has 90% or more homology with the nucleotide sequence of SEQ ID NO:2, and that codes for a nonstructural protein and a structural protein of hepatitis C virus.

2. The recombinant vaccinia virus according to Claim 1, wherein the vaccinia virus is LC16m8 strain.

3. The recombinant vaccinia virus according to Claim 1 or 2, wherein the expression promoter is a hybrid promoter.

4. The recombinant vaccinia virus according to Claim 3, wherein the nucleotide sequence of the hybrid promoter is DNA of (a) or (b) below:

(a) DNA having the nucleotide sequence represented by SEQ ID NO:4; or

(b) DNA that has 90% or more homology with the nucleotide sequence of SEQ ID NO:4, and that has a promoter activity.

5. A pharmaceutical composition comprising the recombinant vaccinia virus according to any one of Claims 1 to 4.

6. The pharmaceutical composition according to Claim 5 as a prophylactic agent for hepatitis C.

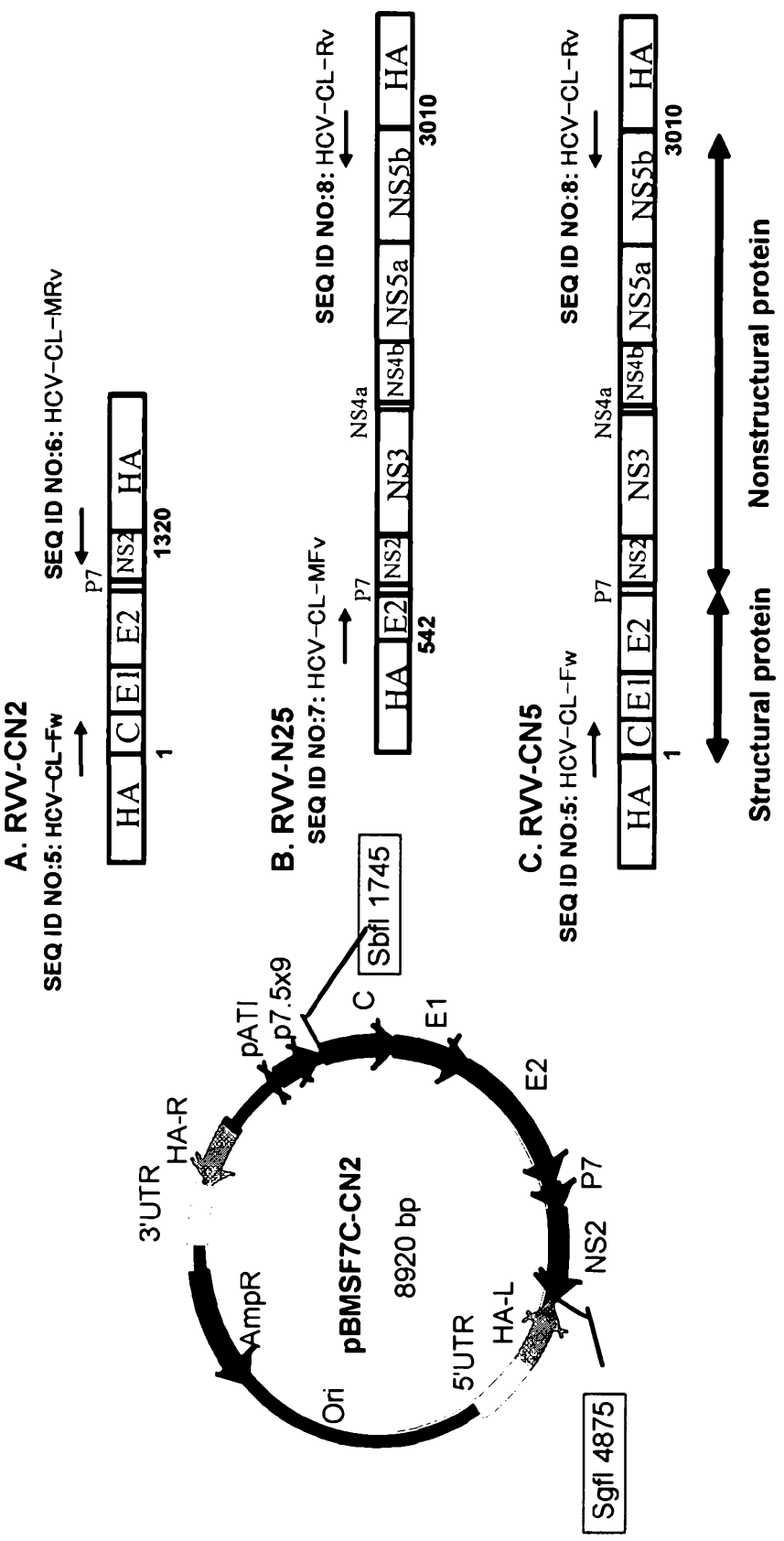
7. The pharmaceutical composition according to Claim 5 as a therapeutic agent for hepatitis C.

8. Use of the recombinant vaccinia virus according to any one of Claims 1 to 4 for the manufacture of a medicament for hepatitis C.

9. A method for treating hepatitis C in a subject in need of treatment, the method comprising administering the recombinant vaccinia virus according to any one of Claims 1 to 4, or the pharmaceutical composition according to any one of claims 5 to 7, to the subject.

10. The recombinant vaccinia virus according to Claim 1, the pharmaceutical composition according to Claim 5, the use according to Claim 8, or the method according to Claim 9, substantially as hereinbefore described.

Fig.1


A. RVV-CN2
SEQ ID NO:5: HCV-CL-Fw
SEQ ID NO:6: HCV-CL-MRv
HA
C
E1
E2
NS2
HA
P7
1
1320
B. RVV-N25
SEQ ID NO:7: HCV-CL-MFv
SEQ ID NO:8: HCV-CL-Rv
HA
E2
NS2
NS3
NS4a
NS4b
NS5a
NS5b
HA
P7
542
3010
C. RVV-CN5
SEQ ID NO:5: HCV-CL-Fw
SEQ ID NO:8: HCV-CL-Rv
HA
C
E1
E2
NS2
NS3
NS4a
NS4b
NS5a
NS5b
HA
P7
1
3010
Structural protein
Nonstructural protein
pBMSF7C-CN2
8920 bp
3'UTR
HA-R
pATI
p7.5x9
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C
E1
E2
P7
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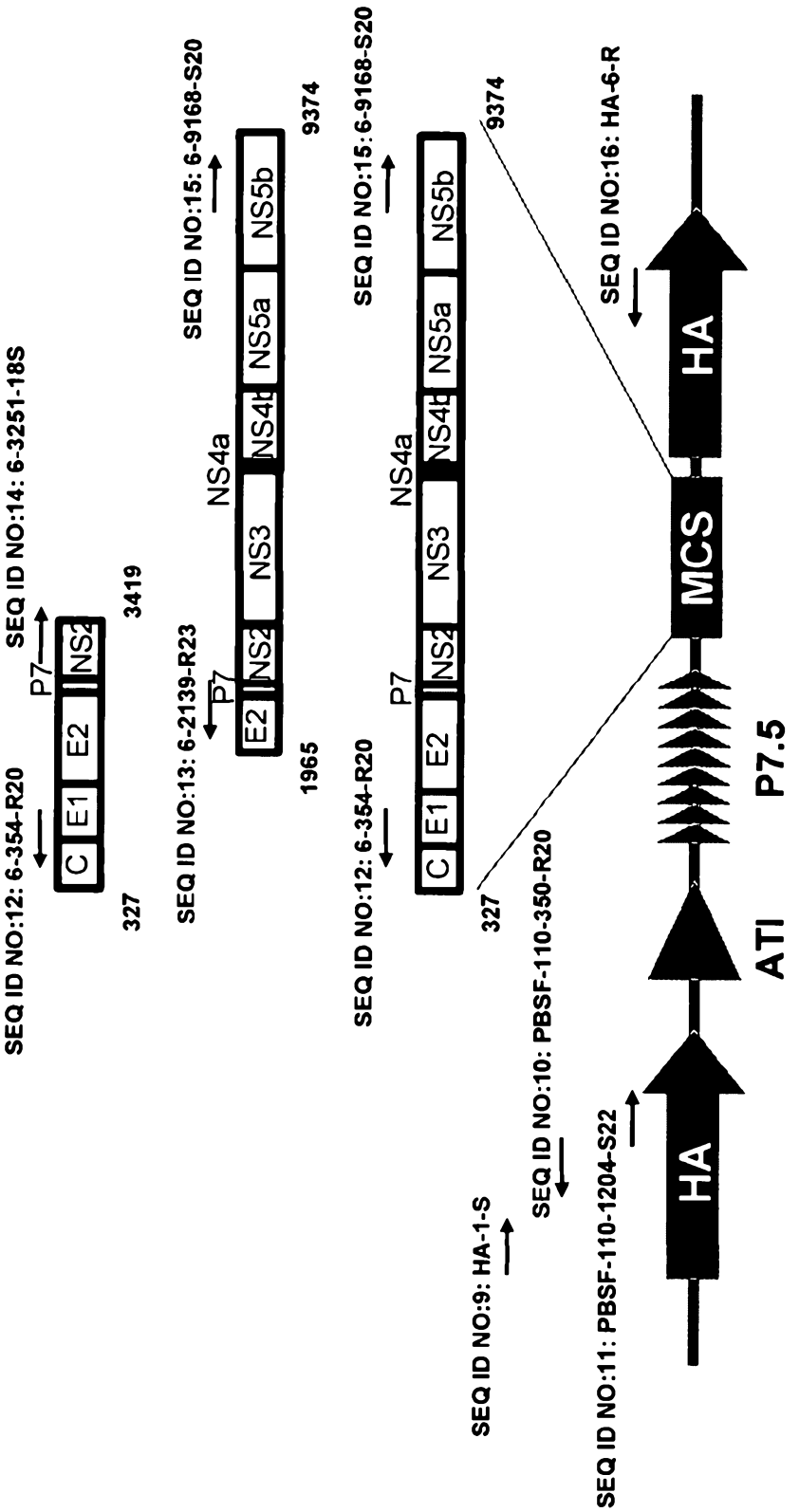
5'UTR
HA-L
Ori
AmpR

Fig.2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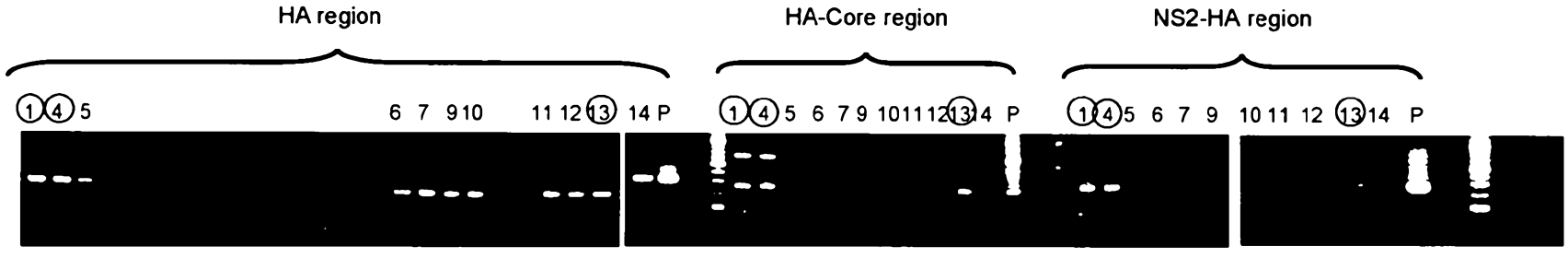
SEQ ID NO:12: 6-354-R20
P7
C
E1
E2
NS2
3419
327
SEQ ID NO:15: 6-9168-S20
SEQ ID NO:13: 6-2139-R23
NS4a
NS3
NS4b
NS5a
NS5b
9374
1965
SEQ ID NO:15: 6-9168-S20
SEQ ID NO:12: 6-354-R20
9374
327
SEQ ID NO:16: HA-6-R
MCS
HA
ATI
P7.5
SEQ ID NO:10: PBSF-110-350-R20
SEQ ID NO:9: HA-1-S
SEQ ID NO:11: PBSF-110-1204-S22

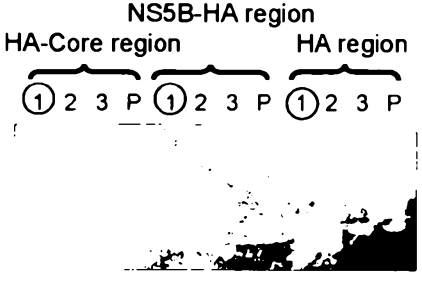
1. RVV-CN2

HA region: SEQ ID NO:9, SEQ ID NO:10
HA-Core region:SEQ ID NO:11, SEQ ID NO:12
NS2-HA region:SEQ ID NO:14, SEQ ID NO:16



1, 4, 13: Clones inserted with the entire gene regions
P: Plasmid vector of positive control

2. RVV-CN5



HA-Core region: SEQ ID NO:11, SEQ ID NO:12
NS5B-HA region:SEQ ID NO:15, SEQ ID NO:16
HA region:SEQ ID NO:9, SEQ ID NO:10

1: Clones inserted with the entire gene regions
P: Plasmid vector of positive control

Fig.4

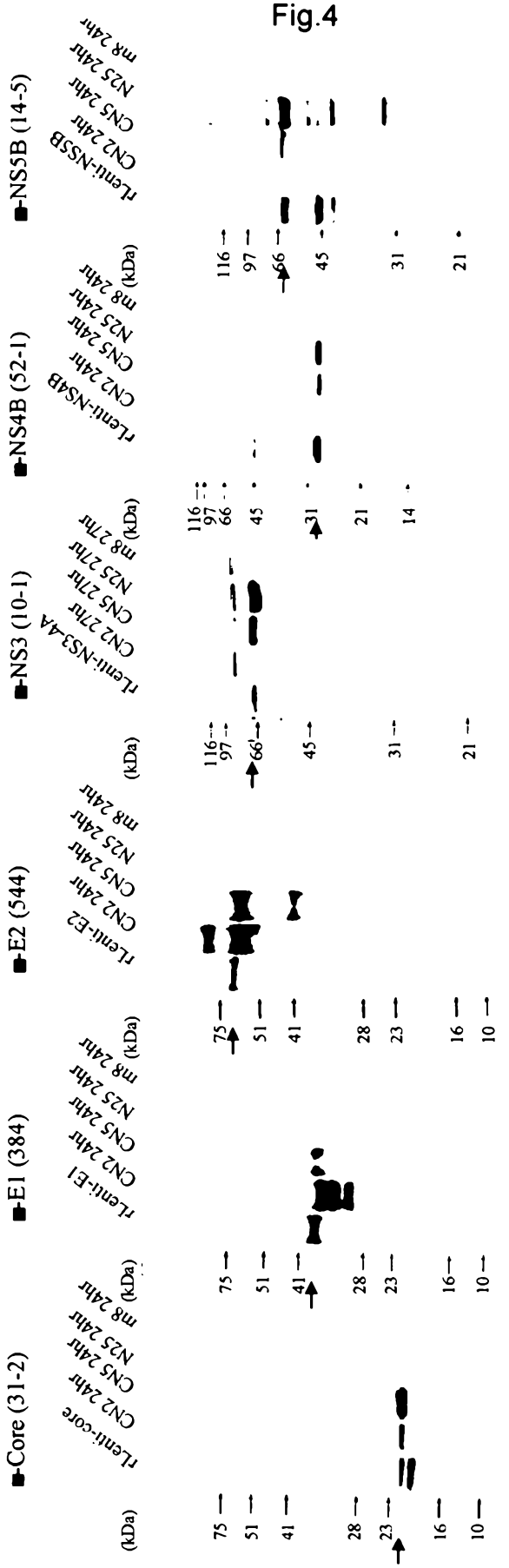
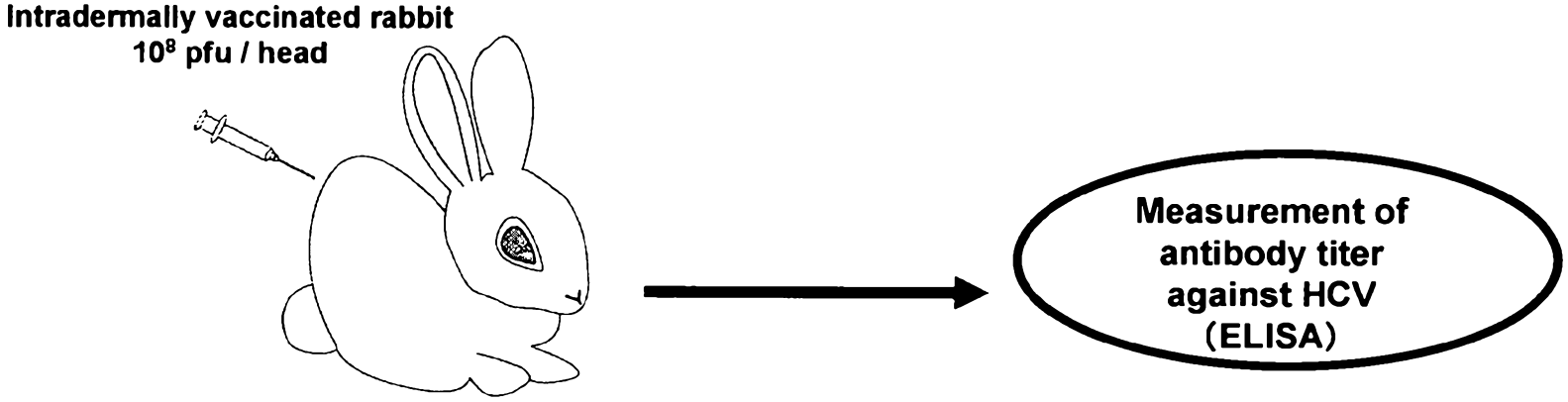

■-Core (31-2)
■-E1 (384)
■-E2 (544)
■-NS3 (10-1)
■-NS4B (52-1)
■-NS5B (14-5)
(kDa)
Flenti-core
Flenti-E1
Flenti-E2
Flenti-NS3-4A
Flenti-NS4B
Flenti-NS5B
CN2 24hr
CN5 24hr
N25 24hr
m8 24hr
CN2 27hr
CN5 27hr
N25 27hr
m8 27hr
75
51
41
28
23
16
10
116
97
66
45
31
21
14

Fig.5

1. Ability of HCV-RVV to induce humoral immunity



2. Ability of HCV-RVV to induce cellular immunity

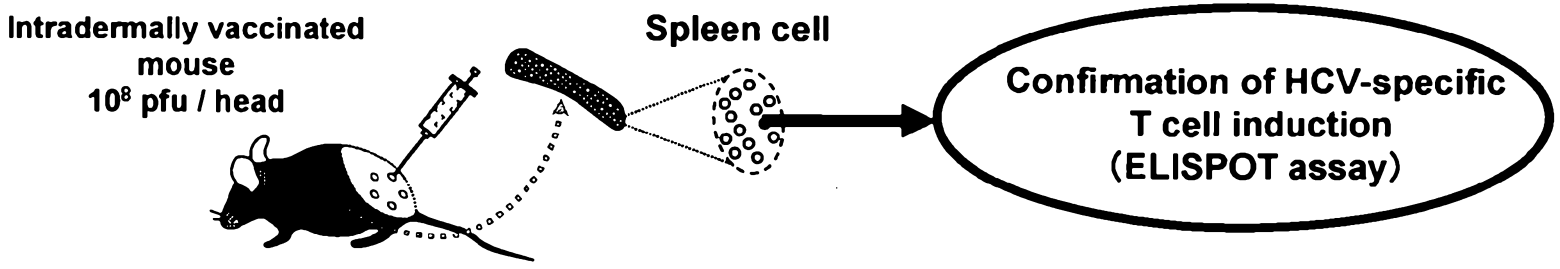


Fig.6

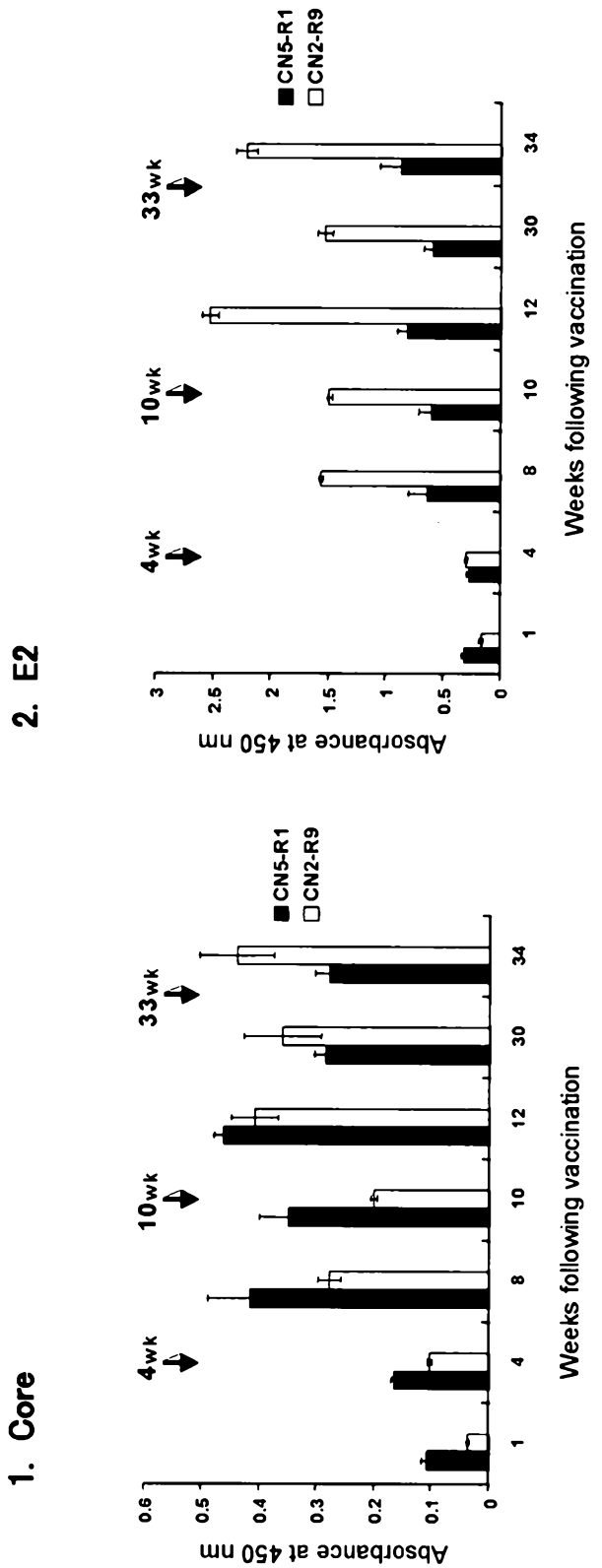
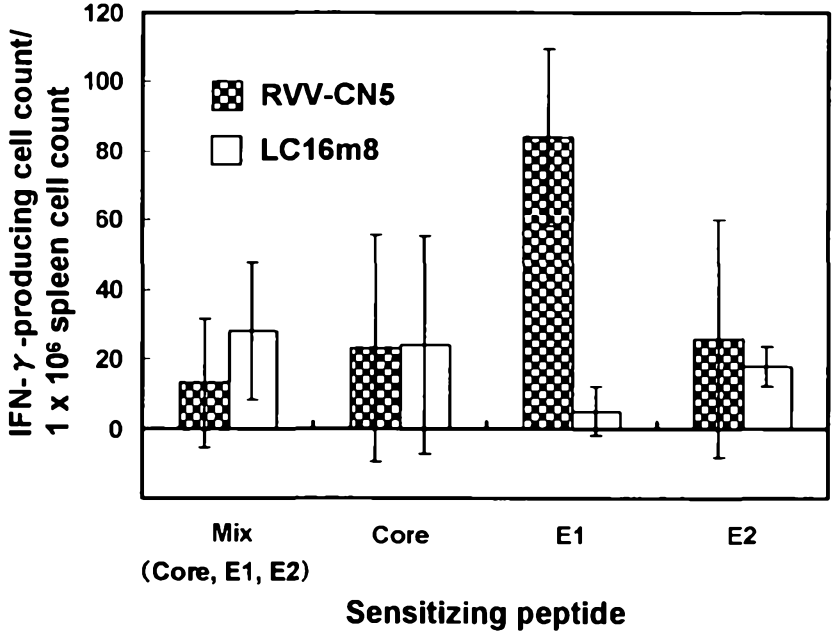

1. Core
2. E2
CN5-R1
CN2-R9
4wk
10wk
33wk
Absorbance at 450 nm
Weeks following vaccination
0
0.1
0.2
0.3
0.4
0.5
0.6
0.5
1
1.5
2
2.5
3
1
4
8
10
12
30
34

Fig.7

1. Amino acid sequences of H-2^d CTL epitope

Region	code	epitope name	References		number of aa	HCR6	
			aa position	sequence		sequence	aa position
Core	1	C7-A10	133-142	LMGYIPLVGA (SEQ ID NO:17)	9	LMGYIPLVGA (SEQ ID NO:17)	133-142
E1	1	E1 315-322	315-322	GHRVAWDM (SEQ ID NO:18)	8	GHRMAYDM (SEQ ID NO:18)	315-322
E2	1	E2 565-574	565-574	GGPPCDIGGV (SEQ ID NO:19)	10	GGPPCNIGGG (SEQ ID NO:20)	565-574

2. Results from ELISPOT assay (BALB/c H-2^d)



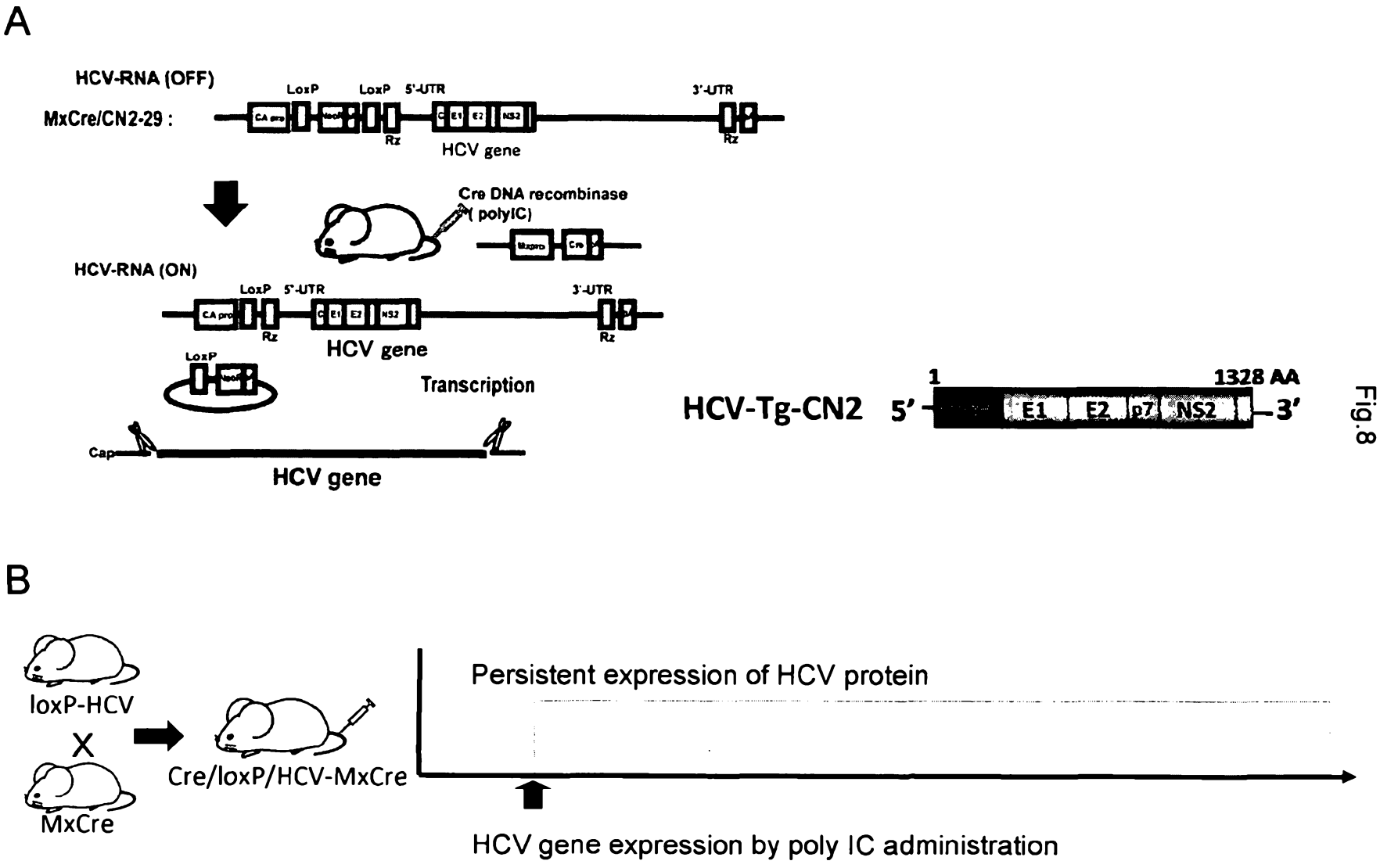

A
HCV-RNA (OFF)
MxCre/CN2-29 :
LoxP
LoxP
5'-UTR
3'-UTR
Rz
HCV gene
Cre DNA recombinase
(polyIC)
HCV-RNA (ON)
Transcription
Cap
HCV gene
HCV-Tg-CN2
1
1328 AA
5'
E1
E2
p7
NS2
3'
B
loxP-HCV
X
MxCre
Cre/loxP/HCV-MxCre
Persistent expression of HCV protein
HCV gene expression by poly IC administration

Fig.8

Fig.9

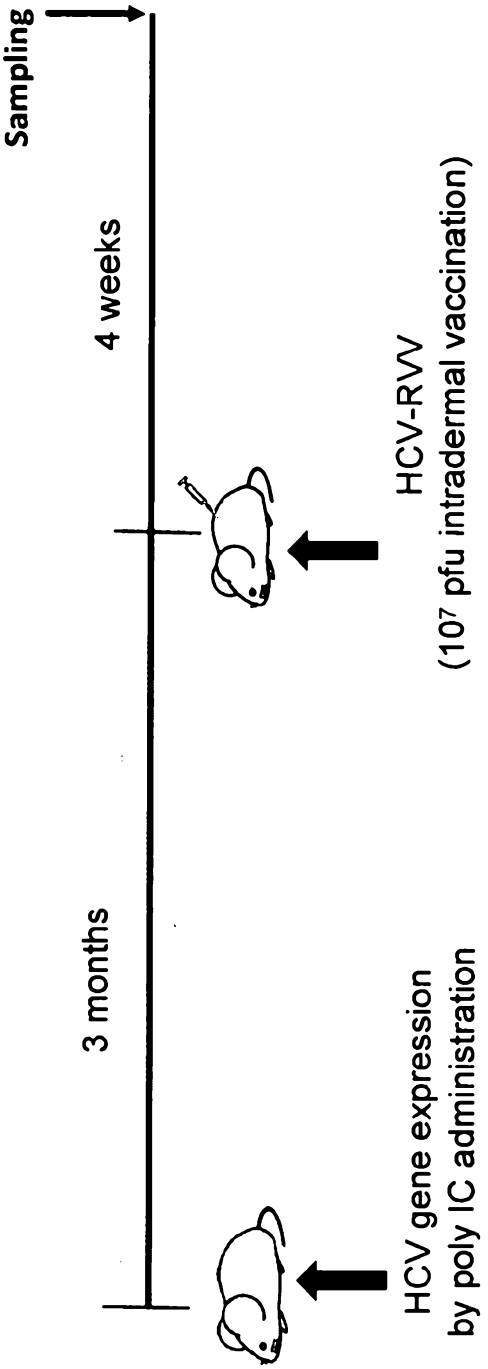
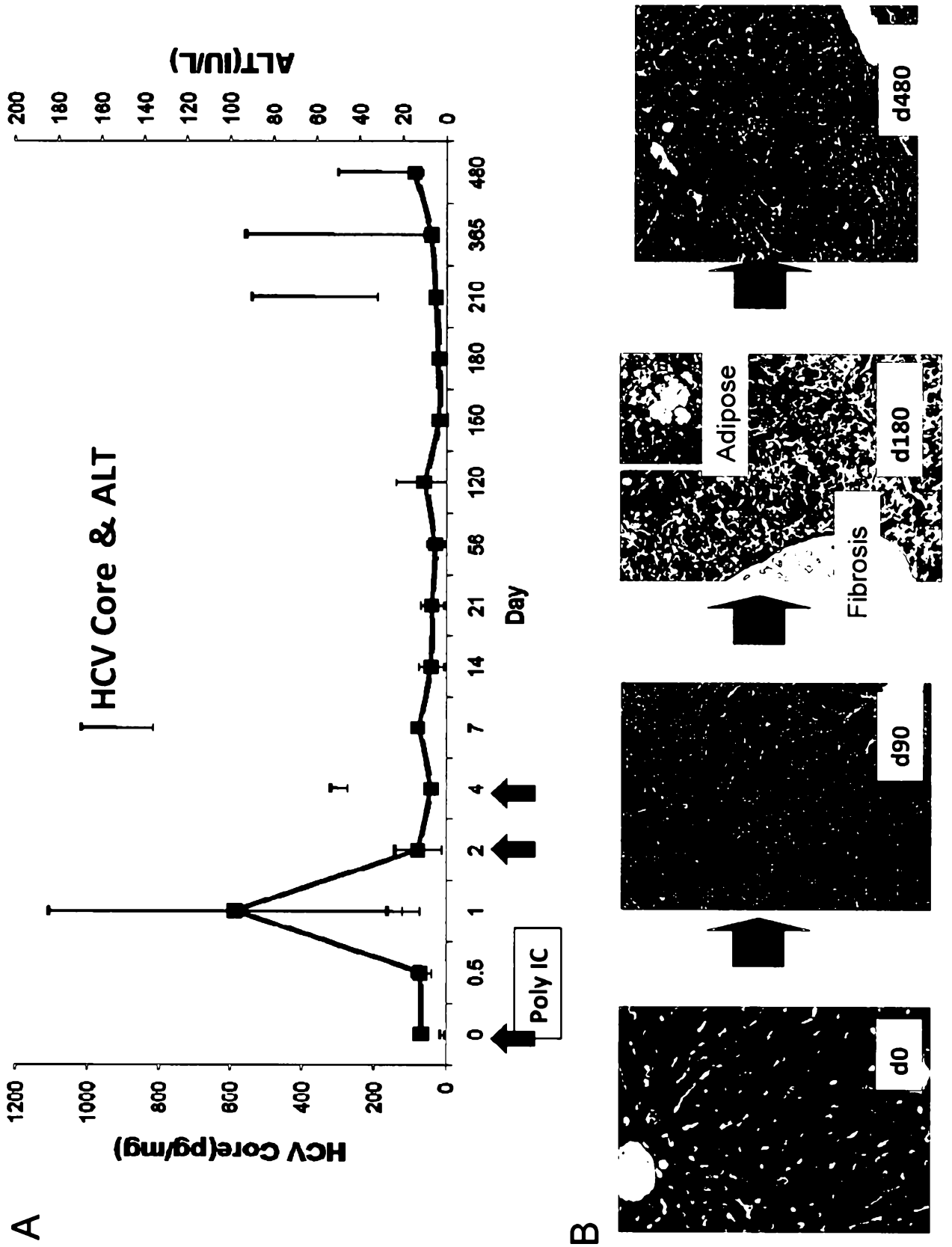
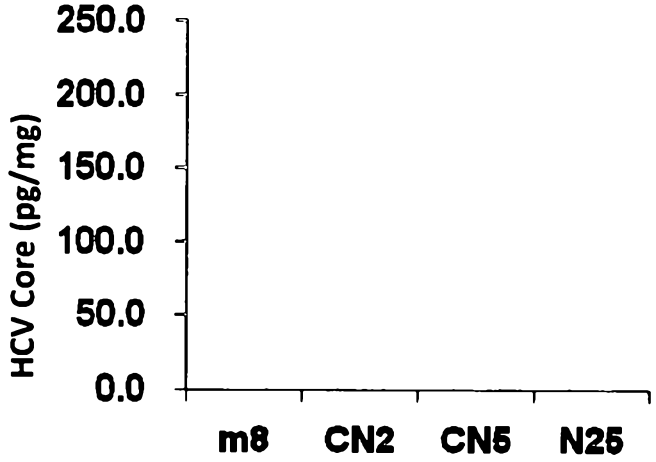

Sampling
4 weeks
HCV-RVV
(10^7 pfu intradermal vaccination)
3 months
HCV gene expression
by poly IC administration

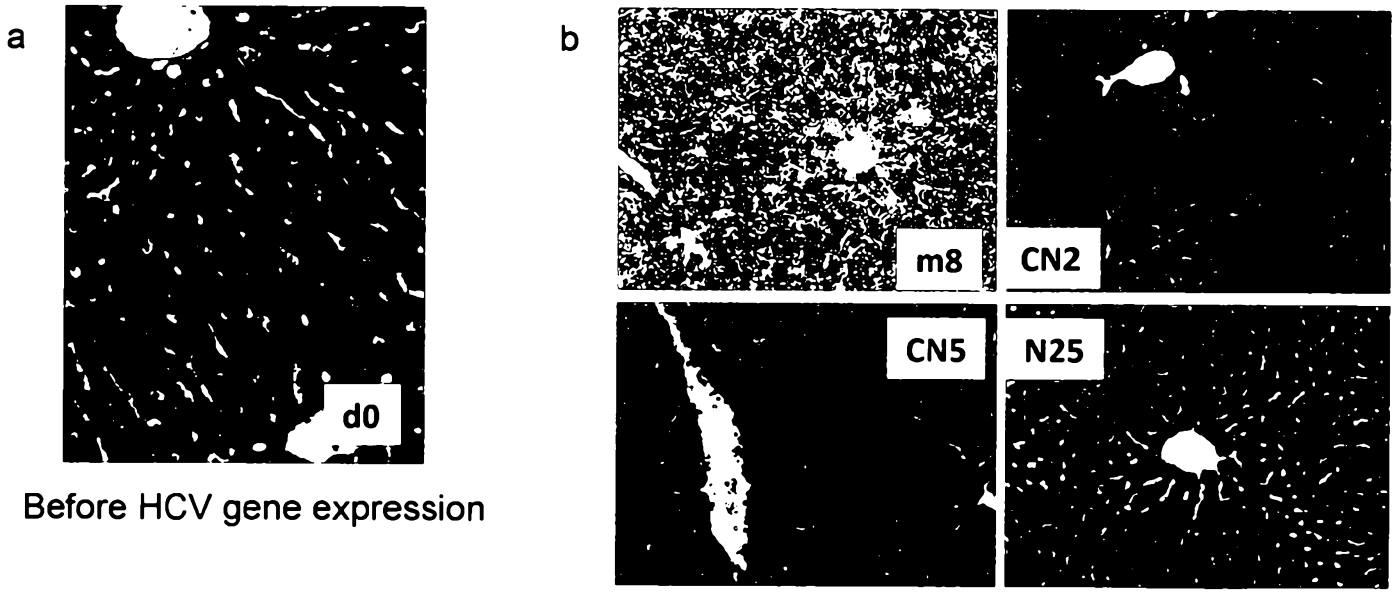
Fig.10


A
HCV Core(pg/mg)
ALT(IU/L)
HCV Core & ALT
Poly IC
Day
0
0.5
1
2
4
7
14
21
56
120
150
180
210
365
480
B
d0
d90
Fibrosis
Adipose
d180
d480

A



B



Before HCV gene expression

Fig.11