The invention relates to a peptidic compound containing a polypeptide NS5b of a hepatitis C virus. Said invention also relates to expression vectors such as adenovirus and poxyvirus in which nucleic sequences coding for the polypeptide NS5b. The inventive compound can be used for a therapeutic application.
Fig. 1C

pTG6624
35117 bps

Fig. 1D

pIV317
37258 bps
Fig. 1E

pTG 4664
5328 bps

Amp-

Fig. 1F

pTG 3074
3240 bps

beta-lactamase
Fig. 2A
Fig. 2B

pTG9997
5054 bps
Fig. 2C
Fig. 2E

pTG186

7296 bps
Fig. 2G

plV329
9499 bps
Fig. 2H

plV344
9499 bps
Fig. 3A

Effector to target cell (E/T) ratios

Fig 3B

Peptide GLL
Irrelevant Peptide
### Fig. 8

<table>
<thead>
<tr>
<th>% Anti-NS3</th>
<th>MFI</th>
<th>% Anti-NS4B</th>
<th>MFI</th>
<th>% Anti-NS5B</th>
<th>MFI</th>
</tr>
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<tbody>
<tr>
<td>Stained cells</td>
<td>86.8</td>
<td>708</td>
<td>Stained cells</td>
<td>86.7</td>
<td>833</td>
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</table>
Fig. 10A

**Schedule 1**
wk 6

**Schedule 2**
wk 5

<table>
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<th>E/T</th>
<th>33:1</th>
<th>11:1</th>
<th>4:1</th>
<th>1:1</th>
<th>0.33:1</th>
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<tr>
<td>GLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% specific Lysis</td>
<td></td>
<td></td>
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</tbody>
</table>

- MVA NS34-NS5B
- MVA N33

<table>
<thead>
<tr>
<th>E/T</th>
<th>33:1</th>
<th>11:1</th>
<th>4:1</th>
<th>1:1</th>
<th>0.33:1</th>
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<td>ALY</td>
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<tr>
<td>% specific Lysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Fig. 10B

Schedule 1
wk 9

% specific Lysis

GLL

33:1 11:1 4:1 1:1 0.33:1

E/T

Schedule 2
wk 8

% specific Lysis

GLL

33:1 11:1 4:1 1:1 0.33:1

E/T

MVA NS34-NS5B

MVA N33

ALY

% specific Lysis

33:1 11:1 4:1 1:1 0.33:1

E/T

ALY

33:1 11:1 4:1 1:1 0.33:1

E/T
Figure 12

<table>
<thead>
<tr>
<th>Spots / 10^6 cells</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
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<td>WPA10</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

MVA NS34-5B

MVA N33
Figure 13

Total splenocytes

Purified CD4+ fraction

Effluent

Spots / 10^6 cells

- MVA NS34-5B
- MVA N33

TT NS3 NS4 NS5B TT NS3 NS4 NS5B TT NS3 NS4 NS5B
Figure 14

![Bar Chart]

- % specific lysis
- MVA NS34-NS5B
- MVA N33

- Wk 5
- Wk 10
- Wk 27
- Wk 29

E/T ratio
Figure 15

15C

- KLT
- KLT median
- Irrelevant
- Irrelevant median

Weeks: Wk5, Wk10, Wk27, Wk29
Figure 16

16A

Spleen

Liver

16B

Spleen

Liver

Log (cfu/mg)

0 1 2 3 4 5 6

MVA NS34-NS5B  TC-LNS3

MVA N33

MVA NS34-NS5B  TC-LNS3

MVA N33

p = 0.02

p = 0.02

p = 0.006

p > 0.05
COMPOSITION COMPRISING THE POLYPROTEIN NS3/NS4 AND THE POLYPEPTIDE NS5B OF HCV, EXPRESSION VECTORS INCLUDING THE CORRESPONDING NUCLEIC SEQUENCES AND THEIR THERAPEUTIC USE

[0001] The present invention relates to the field of prophylactic and therapeutic vaccination directed against the hepatitis C virus (HCV). It relates in particular to a novel composition containing a polyprotein corresponding to the two collinear proteins NS3 and NS4 (hereafter called polyprotein NS3/NS4) and a polypeptide constituted by NS5b, the vectors, such as adenovirus or poxvirus, capable of expressing this composition and their use as vaccine.

[0002] Hepatitis C is the major cause of transfusion-acquired hepatitis. Hepatitis C can also be transmitted by other percutaneous routes, for example by injection of drugs by intravenous route. The risk of contamination of health professionals is moreover not negligible. Sexual transmission has been described.

[0003] Hepatitis C differs from other forms of liver diseases associated with viruses, such as hepatitis A, B or D. The infections by the hepatitis C virus (HCV or HCV) are mostly chronic resulting in diseases of the liver, such as hepatitis, cirrhosis and carcinoma in a large number of cases (5 to 20%) and represents 30% of the hepatic transplants in developed countries.

[0004] Although the risk of transmission of the virus by transfusion has diminished owing to the introduction of screening tests in the 1990s, the frequency of new HCV infections remains high. By way of example, a recent study indicates that today there are still 10,000 to 15,000 new cases of infection per year in France (S. Deuflic et al., Hepatology 1999; 29: 1596-1601). Currently, approximately 170 million people worldwide are chronically infected by HCV (Hepatitis C: Global prevalence (update), 2000. Weekly Epidemiological Record, Vol 75(3)). The high-risk populations are principally hospital staff and intravenous-drug users, but there are asymptomatic blood donors who do not belong to these high-risk groups and in whom circulating anti-HCV antibodies have been found. For the latter, the infection route has not yet been identified. HCV infections therefore exist (estimated at between 5 and 10%), known as sporadic infections, the etiology of which is unknown and which cannot be controlled.

[0005] HCV was the first hepatotropic virus isolated by means of molecular biology techniques. The viral genome sequences were cloned before the viral particle was visualized.

[0006] HCV belongs to a new genus of the Flaviviridae family, the hepaciviruses. It is a positive single-strand RNA virus, of 9.5 kb, which is replicated by a complementary RNA copy and the translation product of which is a polyprotein precursor of approximately 3,000 amino acids. The 5' end of the HCV genome corresponds to an untranslated region and the gene core are contiguous proteins (Brinster et al., 2001, Hepatology, 34: 1206-1217), or by a mixture of structural and non-structural genes that code for the non-structural proteins (NS2, NS3, NS4, NS5) and for a 3' non-coding region possessing a well-conserved domain (Major M E, Feinstone S M, Hepatology, June 1997, 25 (6): 1527-1538).

[0007] At present, the most effective therapy for the treatment of hepatitis C combines pegylated interferon and ribavin (Manns M P et al., The Lancet, 22 Sep. 2001, Vol. 358, 958-965). Whilst this therapy is particularly effective in the case of patients infected by viral strains belonging to the genotypes 2 and 3, it still has only a limited effect on the genotypes 1a, 1b and 4 (Manns M P, op. cit.). Less than 50% of the treated patients become "long-term responders". Moreover, this therapy is an expensive intervention (10,000 to 15,000 euros/patient/year) and is associated with toxic effects. In fact, 5 to 10% of the patients are obliged to stop treatment before the end.

[0008] It is therefore necessary to develop a vaccine composition targeting all the genotypes.

[0009] Several studies now show that the control of an infection caused by HCV either naturally (spontaneous resolution), or after treatment (therapeutic resolution) is associated with the induction or potentialization of cell-mediated immune responses involving the T-CD4* and T-CD8* lymphocytes (as described for example in LECHNER, F. et al., Eur. J. Immunol., 30: 2479-2487 (2000) and in Thimme R. et al., 2001, J. Exp. Med., 194 (10): 1395-1406).

[0010] The molecules of the major histocompatibility complex (MHC, also known as HLA in humans) are referred to as class I or class II. The class I molecules are expressed on virtually all of the nucleated cells and are able to present epitopes or peptides to the CD8* cytotoxic T lymphocytes (CTL). The class II molecules are able to present epitopes to the CD4* T cells, but their expression is restricted to antigen-presenting cells.

[0011] The vaccines against the hepatitis C virus currently envisaged are based on the use of adjuvant recombinant proteins, peptides, expression vectors among which there can be mentioned vectors of viral or bacterial origin or of naked DNA. In this case, one or more viral proteins or one or more genes coding for these viral proteins are used.

[0012] When several viral proteins or one or more genes coding for these viral proteins are selected, the latter are often constituted either by some or all of the structural proteins (Makimura et al., 1996, Vaccine, 14: 28-34; Fourzy J. et al., 1996, J. Virol., 70: 6477-6484), by individual non-structural proteins or comprising at least two contiguous proteins (Brinster et al., 2001, Hepatology, 34: 1206-1217), or by a mixture of structural and non-structural proteins (Pancholi et al., 2003, J. Virol., 77: 382-390).

[0013] The Patent Application WO99/38880 describes the use of three genes coding separately for the three proteins NS3, NS4 and NS5 (a and b) in a vaccine composition comprising three DNA vaccines each expressing these three proteins separately. The authors show the induction of T lymphocytes specific to the three antigens in mice. Only the vaccine expressing NS5a and b has been tested in vivo in a protection test.
[0014] The Patent Application WO01/30812 describes the use of a fusion protein constituted by the non-structural proteins NS3, NS4 and NS5a, if necessary in combination with the non-structural protein NS5b. The authors have indicated that this combination made it possible to activate the HCV-specific T cells. This patent application simply describes the ability of vaccine formulations (naked-DNA, recombinant-adenovirus or recombinant-vaccinia-virus type) expressing the fusion protein NS3, NS4, NS5a or the protein NS5b to induce specific immune responses mediated by specific T lymphocytes.

[0015] The Applicant has now demonstrated, against all expectation, that the particular combination of the non-structural proteins NS3, NS4 and NS5b, NS3 and NS4 being expressed colinearly had a better immunogenic power and protective power superior to that obtained with a vaccine also including, apart from these non-structural proteins, the protein NS5a and/or other structural proteins of HCV such as core, E1 or E2, and had an effect on the ability of cells originating from patients infected by viral strains to induce specific immune responses.

[0016] Thus, an object of the present invention is a peptide composition comprising a polyprotein NS3/NS4 of the hepatitis C virus, as well as a polypeptide NS5b of the hepatitis C virus.

[0017] An object of the invention is also the vectors including the nucleotide sequences coding for this peptide composition, such as the adenoviruses and poxviruses, as well as microorganisms or host cells transformed by these vectors.

[0018] An object of the invention is also a pharmaceutical composition comprising as active ingredient the peptide composition, vector(s) or antibodies of the invention and a pharmaceutically acceptable vehicle.

[0019] An object of the invention is also antibodies directed against the peptide composition of the invention, as well as the use of the peptide composition, vectors, pharmaceutical composition or antibodies for the preparation of a medicament intended for the inhibition or control of an infection caused by the hepatitis C virus, and in a vaccine composition.

[0020] Another object of the invention is a method of treatment of one or more pathologies associated with an hepatitis C virus, which comprises at least one administration to a host organism of an effective dose of any of the above-described active ingredient (peptide composition, vector, antibodies and/or pharmaceutical composition) or any combination thereof.

[0021] An object of the invention is finally a method of stimulating a cell-mediated immune response against an hepatitis C virus protein which comprises the step of administering in a host organism at least one dose of the peptide composition, vector, pharmaceutical composition or antibodies.

[0022] As used herein throughout the entire application, the terms “it” and “an” are used in the sense that they mean “at least one”, “at least a first”, “one or more” or “a plurality” of the referenced compounds or steps, unless the context dictates otherwise. For example, the term “a cell” includes a plurality of cells including a mixture thereof.

[0023] The term “and/or” wherever used herein includes the meaning of “and”, “or” and “all or any other combination of the elements connected by said term”.

[0024] The term “about” or “approximately” as used herein means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

[0025] As used herein, when used to define products, compositions and methods, the term “comprising” is intended to mean that the products, compositions and methods include the referenced components or steps, but not excluding others. “Consisting essentially of” shall mean excluding other components or steps of any essential significance. Thus, a composition consisting essentially of the recited components would not exclude trace contaminants and pharmaceutically acceptable carriers. “Consisting of” shall mean excluding more than trace elements of other components or steps. For example, a polypeptide “consists of” an amino acid sequence when the polypeptide does not contain any amino acids but the recited amino acid sequence. A polypeptide “consists essentially of” an amino acid sequence when such an amino acid sequence is present together with only a few additional amino acid residues, typically from about 1 to about 50 or so additional residues. A polypeptide “comprises” an amino acid sequence when the amino acid sequence is at least part of the final amino acid sequence of the polypeptide. Such a polypeptide can have a few up to several hundred additional amino acids residues. Such additional amino acid residues may play a role in polypeptide trafficking, facilitate polypeptide production or purification; prolong half-life, among other things. The same can be applied for nucleotide sequences.

[0026] The present invention therefore proposes a novel peptide composition constituted by a polypeptide NS3/NS4 and a polypeptide NS5b of HCV, which composition has the ability to stimulate a cell-mediated immune response specific to HCV, such that it is useful in the field of prophylactic and therapeutic vaccination directed against the hepatitis C virus.

[0027] The polypeptide NS3/NS4 of the peptide composition of the invention is constituted by the protein NS3 and the protein NS4a and b, without interruption in the peptide sequence, as in the native polypeptide. In fact, as indicated previously, the HCV genome contains a single open reading frame that is transcribed into a polypeptide. This HCV polypeptide can be cleaved in order to produce at least ten distinct parts, in the order N1L-Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH.

[0028] For general guidance, the protein NS3 is a protein of 630 amino acids, which appears approximately from amino acid 1027 to amino acid 1657 of the polypeptide. The protein NS4, a protein of 314 amino acids, appears approximately from amino acid 1658 to amino acid 1972 (numbering with respect to HCV-1) (Choo et al., 1991, Proc. Natl. Acad. Sci., vol 88: 2451-2455). The polypeptide NS3/NS4 therefore appears approximately from amino acid 1027 to amino acid 1972.

[0029] As regards the polypeptide NS5b also contained in the composition of the invention, it is constituted by 590 amino acids and appears approximately from amino acid 2421 to amino acid 3011 of the polypeptide (Choo et al., 1991, op. cit.).
For sake of clarity, the amino acid stretches referred herein in connection with NS3, NS4 and NS5B proteins are given with respect to their positions in HCV-1 polyprotein precursor (as described by Choo et al., 1991, Proc. Natl. Acad. Sci. USA 88, 2451-2455 or in GenBank under accession number M62321). However, the present invention also encompasses NS3, NS4 and NS5B proteins of other HCV strains and isolates, as well as analogues or muteins thereof.

The protein NS3 comprises two distinct structural domains, namely an N-terminal domain endowed with an active serine protease activity that is involved in the maturation of the viral polyprotein, and a C-terminal domain comprising a helicase activity associated with an NTPase activity that plays a role in the replication of the viral genome.

By “polyprotein NS3/NS4” and “polypeptide NS5b”, is of course meant the polypeptides and polypeptides having the native amino acid sequences, originating from any HCV strain and isolate, as well as their analogues, muteins and homologues.

By “analogue” or “mutein” of the polyprotein and of the polypeptide, is meant the biologically active derivatives of the reference molecules that have the desired activity, namely the ability to stimulate a cell-mediated immune response as defined above.

Generally, the term “analogue” refers to compounds having a native polypeptide sequence and structure having one or more additions, substitutions (generally conservative in nature and/or amino acid deletions, relative to the native molecule, to the extent that the modifications do not destroy the immunogenic activity. By the term “mutein”, is meant the peptides having one or more elements imitating the peptide (peptoids), such as those described in the Patent Application PCT W091/04282. Preferably, the analogue or the mutein have at least the same immunoactivity as the native molecule. Processes for preparing polypeptide analogues and muteins are known to a person skilled in the art and are described below.

The particularly preferred analogues include substitutions that are conservative in nature, i.e. the substitutions, which take place in a family of amino acids. Specifically, the amino acids are generally divided into 4 families, namely (1) the acid amino acids such as aspartate and glutamate, (2) the basic amino acids such as lysine, arginine and histidine, (3) the non-polar amino acids such as alanine, leucine, isoleucine, proline, phenylalanine, methionine and tryptophane and (4) the polar non-charged amino acids such as glutamate, asparagine, glutamine, cysteine, serine, threonine and tyrosine. Phenylalanine, tryptophane and tyrosine are sometimes classified as aromatic amino acids. For example, it can reasonably be predicted that an isolated replacement of leucine by isoleucine or valine, of an aspartate by a glutamate, of a threonine by a serine, or a similar conservative replacement of one amino acid by another amino acid having a structural relationship, will not have a major effect on the biological activity. A person skilled in the art will easily determine the regions of the peptide molecule of interest that can tolerate a change by referring to the Hopp/ Woods and Kyte-Doolittle plots, well known in the art.

By “homology”, is meant the percentage of identity between two peptide molecules, such as polypeptides and polypeptides. Two amino acid sequences are “more or less homologous” to each other when the sequences have at least 60%, preferably at least 75%, more preferably also at least 80-85%, more preferably also at least 90% and still more preferably at least 95-98% or more of sequence identity over a defined length of the peptide molecules.

Generally, the term “identity” refers to an exact amino acid to amino acid correspondence of two peptide sequences. The percentage of identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of mismatches between the two aligned sequences, dividing by the length of the shorter sequence and multiplying the result by 100. The percentage of identity can also be determined using computer programs such as ALIGN, Dayhoff, M. O. in Atlas of Protein Sequence and Structure M. O. Dayhoff, 1981, 5 Suppl., 3: 482-489.

The nucleic acid and amino acid sequences of a certain number of HCV strains and isolates, and in particular of the protein NS3, of the protein NS4 and of the polypeptide NS5b, have already been determined.


The HCV strains and isolates, as illustrated above, have different genotypes, namely genotypes 1a (isolates HCV-1, -J1 and -H), 1b (isolates HCV-J and BK), 2c (isolate HCV-NS5A), 2a (isolate HCV-NS5B), 3a (isolate HCV-NS5C), 3b (isolate HCV-NS5D), 4a (isolate HCV-ED43), 5a (isolate HCV-EUH1480), 6a (isolate HCV-NS5E), 7b (isolate HCV-NS5F), 8b (isolate HCV-NS5G), 9a (isolate HCV-NS5H), 10a (isolate HCV-JK049) and 11a (isolate HCV-JK046).
[0041] According to one embodiment of the invention, NS3 and/or NS4 and/or NS5b originate from viruses of different genotypes. For example, the NS3/NS4 polyprotein and the NS5b polypeptide can originate from viruses of different genotypes, e.g. NS3/NS4 originating from a genotype 1b and NS5b form a genotype 4 or vice versa.

[0042] According to another embodiment, NS3 and/or NS4 and/or NS5b originate from viruses of the same genotype, preferably of genotype 1b. A preferred embodiment of the present invention is directed to a peptide composition comprising a polyprotein NS3/NS4 as well as a polypeptide NS5b originating from the genotype 1b HCV JA strain (Kato et al., 1990, Proc. Natl. Acad., Sci. 87, 9524-9528). More preferably, the polyprotein NS3/NS4 comprises, or alternatively consists essentially or alternatively consists of the amino acid sequence shown in SEQ ID NO: 2 and/or the NS5b polypeptide comprises or alternatively consists essentially or alternatively consists of the amino acid sequence shown in SEQ ID NO: 4.

[0043] The polyprotein NS3/NS4 and the polypeptide NS5b contained in the peptide composition of the invention can be either of native origin, or of recombinant origin.

[0044] The polyprotein NS3/NS4 and the polypeptide NS5b of native origin are obtained from HCV strains or isolates, by means of the use of synthetic oligonucleotide primers that will serve to amplify the native viral sequences, either from sera of patients infected by the targeted viral genotype or genotypes, or from already purified viral RNA, originating for example from patients’ blood or liver, or from complementary DNA that is free or cloned beforehand in an expression vector, or also from viral particles purified from biological samples or in vitro propagation system.

[0045] The polyprotein NS3/NS4 and the polypeptide NS5b of the invention of recombinant origin can also be obtained by the genetic engineering technique, which comprises the steps of:

[0046] culture of a microorganism or of eukaryotic cell(s) transformed using a nucleotide sequence coding for said polyprotein NS3/NS4 or for said polypeptide NS5b and

[0047] recovery of the peptide produced by said microorganism or said eukaryotic cells.

[0048] This technique is well known to a person skilled in the art. For more details concerning this, reference can be made to the following work: Recombinant DNA Technology I, Editors Ales Prokop, Raskesh K Bajpai; Annals of the New-York Academy of Sciences, Volume 646, 1991.

[0049] The nucleotide sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b can be prepared by chemical synthesis in conjunction with a genetic engineering approach or by genetic engineering alone, using the techniques well known to a person skilled in the art and described for example in Sambrook J. et al., Molecular Cloning: A Laboratory Manual, 1989.

[0050] The nucleotide sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b can be inserted into expression vectors in a suitable expression system, in order to obtain the peptide composition of the invention.

[0051] The term “expression vector” as used herein refers to viral as well as non viral vectors, including extrachromosomal vectors (e.g. multicopy plasmids) and integrating vectors designed for being incorporated into the host chromosome(s). Particularly important in the context of the invention are vectors for use in gene therapy which are capable of delivering the NS3/NS4 and NS5b-encoding nucleotide sequences to a host organism as well as expression vectors for use in various expression systems. When referring to a “viral vector”, this term encompasses any vector that comprises at least one element of viral origin, including a complete viral genome, a portion thereof or a modified viral genome as described below as well as viral particles generated thereof (e.g. viral vector packaged into a viral capsid to produce infectious viral particles).

[0052] Of course, the nucleotide sequences can be inserted into a single expression vector or into two different expression vectors. In the latter case, the sequence coding for the polyprotein NS3/NS4 is inserted into one of the two vectors and the sequence coding for the polypeptide NS5b is inserted into the other vector, these two vectors being either identical or different in nature.

[0053] Thus, another object of the invention is the expression vector(s) comprising a nucleotide sequence coding for the polyprotein NS3/NS4 and a nucleotide sequence coding for the polypeptide NS5b, as well as the means necessary to its expression.

[0054] By means necessary to the expression of a peptide are meant, the term peptide being used for any peptide molecule, such as protein, polyprotein, polypeptide, etc., any means that make it possible to obtain the peptide, such as in particular a promoter, a transcription terminator, a replication origin and preferably a selection marker.

[0055] The means necessary to the expression of a peptide are operationally linked to the nucleotide sequence coding for the peptide of interest. By “operationally linked”, it means a juxtaposition of said elements necessary to the expression and of the gene coding for the peptide of interest, which are in a relationship such that it is possible for them to function in an expected manner. For example, additional bases can exist between the promoter and the nucleotide sequence to the extent that their functional relationship is preserved.

[0056] The means necessary to the expression of a peptide can be homologous means, i.e. included in the genome of the vector used, or be heterologous. In the latter case, said means are cloned with the peptide of interest to be expressed.

[0057] Examples of heterologous promoters include (i) the viral promoters such as the SV40 promoter (simian virus 40), the promoter of the thymidine-kinase gene of the herpes simplex virus (TK-HSV-1), the LTR of the Rous sarcoma virus (RSV), the immediate first promoter of the cyto-megalovirus (CMV), the adenovirus major last promoter (MLP), as well as (ii) any cell promoter that controls the transcription of the genes coding for peptides in upper eukaryotes, such as the constitutive promoter of the diphosphoglycerate kinase gene (PGK) (Adra et al., 1987, Gene, 60: 65-74), the promoter of the liver-specific alpha-1 antitrypsin and FIX genes and the SM22 promoter specific to the smooth muscle cells (Moessler et al., 1996, Development, 122: 2415-2425).
purposes of the invention, there can be mentioned for promoters and/or regulators that are independent identical or promoter and/or of a single expression-regulating element, comprises or alternatively consists essentially of, or alternatively consisting of the sequence as shown in SEQ ID NO: 1 and the nucleotide sequence which encodes the polypeptide NS5b comprises or alternatively consists essentially of, or alternatively consists of a sequence as shown in SEQ ID NO: 3.

Here too, by “nucleotide sequence” is meant all the sequences coding for the native polynucleotide NS3/NS4 and the native polynucleotide NS5b, as well as for their analogues, muteins and homologues, as defined previously.

Said sequences contained in the expression vector can be directly interlinked under the control of a single promoter and/or of a single expression-regulating element, or they can be separate, each being dependent on expression promoters and/or regulators that are independent identical or different.

As expression vectors that are suitable for the purposes of the invention, there can be mentioned for example plasmids, adenovirus-type viral vectors, poxviruses, vaccinia viruses, baculoviruses, salmonella-type bacterial vectors, BCG.

Adenoviruses have been detected in numerous animal species, do not integrate and are only slightly pathogenic. They are capable of infecting a variety of cell types, cells in division and cells at rest. They possess a natural tropism for the bronchial epithelia. Moreover, they have been used as live enteric vaccines for many years with an excellent safety profile. Finally, they can easily be made to grow and be purified in large amounts. These characteristics have meant that the adenoviruses are particularly appropriate for use as expression vectors and in particular as gene therapy vectors for therapeutic purposes and for vaccines.

According to a preferred embodiment, the vector of the invention is an adenovirus.

Examples of adenoviruses to be used in the present invention can be derived from any source of human or animal origin, in particular of canine origin (for example CAV-1 or CAV-2; reference Genbank CAV1GENOM and CAV77082 respectively), of avian origin (reference Genbank AAEDSDNA), of bovine origin (such as BAV3, Seshidhar Reddy et al., 1998, J. Virol., 72: 1394-1402), of ovine, feline, porcine origin, of simian origin, or from one of their hybrids. Any serotype can be used. However, adenoviruses of human origin are preferred and in particular adenovirus 5 (Ad5), adenovirus-2 (Ad2) and adenovirus-35 (Ad35).

Generally, the mentioned viruses are available from the ATCC collections and have been the subject of numerous publications describing their sequence, their organization and their biology, which allows a person skilled in the art to use them easily. For example, the sequence of the adenovirus type 5 is described in the Genbank database (M73260 and M29978) and is incorporated here by way of reference.

The genome of the adenovirus is constituted by a double-strand linear DNA molecule of approximately 36 kb carrying more than approximately 30 genes necessary for terminating the viral cycle. The first genes are divided into 4 regions dispersed in the genome of the adenovirus (E1 to E4). The E1, E2 and E4 regions are essential for viral replication. The E3 region is considered as a non-essential region on the basis of the observation that mutant viruses appear naturally or the hybrid viruses having lost this E3 region continue to replicate like wild-type viruses in cultured cells (Kelly and Lewis, 1973, J. Virol., 12: 643-652). The late genes (L1 to L5) mostly code for the structural proteins constituting the viral capsid. They overlap at least in part the first transcription units and are transcribed from a single promoter (MLP for Major Late Promoter). Moreover, the adenoviral genome carries at the two ends of the cis-acting regions essential for DNA replication, the 5' and 3' inverted terminal repeats (ITRs) and a packing sequence respectively.

The adenoviruses currently used in gene therapy protocols are stripped of the majority of the E1 region, which renders the viruses deficient at the level of their replication in order to avoid their dissemination in the environment and in the host organism. Moreover, most of the adenoviruses are also stripped of the E3 region in order to increase their cloning capacity. The feasibility of gene transfer using these vectors has been demonstrated in a variety of tissues in vivo (see for example Yei et al., 1994, Hum. Gene Ther., 5: 731-744; Dai et al., 1995, Proc. Natl. Acad. Sci. USA, 92: 1401-1405; U.S. Pat. No. 6,099,831; and U.S. Pat. No. 6,013,638).

Preferably also, the CMV promoter is the promoter of the polypeptide NS3/NS4 and the expression vector comprises as nucleotide sequence coding for said polypeptide the expression cassette CMV-NS3-NS4.

By “expression cassette”, is meant a DNA sequence containing a promoter and an open reading frame for the expression of the peptide of interest, to be inserted into a vector.

Preferably also, the SV40 promoter is the promoter of the polypeptide NS5b and the expression vector comprises as nucleotide sequence coding for said polypeptide the expression cassette SV40-NS5b.

According to one embodiment of the invention, the genome of the adenovirus is modified so as to replace the E3 region by the expression cassette CMV-NS3-NS4 and to replace the E1 region by the expression cassette SV40-NS5b.

The methods of suppression and of insertion of DNA sequences into expression vectors are widely known to a person skilled in the art and consist in particular of steps of enzymatic digestion and ligation or homologous recombination (Chartier et al., 1996, J. Virol. 70, 4805-4810).
Another expression vector particularly appropriate for the purposes of the invention is a poxvirus, which constitutes another embodiment of the invention.

The poxviruses constitute a group of enveloped complex viruses, differing principally in their unusual morphology, their large DNA genome and their cytoplasmic replication site. The genome of several elements of the poxviridae, comprising the Copenhagen strain of the vaccinia virus (VV) (Goebel et al., 1990, Virol. 179: 247-266 and 517-563) and the modified vaccinia virus Ankara (MVA) strain (Antoine et al., 1998, Virol., 244: 635-396), has been mapped and sequenced. The VV strain possesses a double-strand DNA genome of approximately 192 kb coding for approximately 200 proteins approximately 100 of which are involved in the assembly of the virus. The MVA strain is a highly attenuated strain of vaccinia virus, generated by more than 500 passages in series of the vaccinia virus Ankara strain (CVA) over chicken embryo fibroblasts (Mayr et al., 1975, Infection, 3: 6-16). The MVA virus has been deposited in the Collection Nationale de Cultures de Micro-organisms (CNMC) under Number 1-721. The determination of the complete sequence of the MVA genome and comparison with that of the W allows precise identification of the alterations that have appeared in the viral genome and the definition of seven deletions (I to VII) and of numerous mutations leading to fragmented open reading frames (Antoine et al., 1998, Virol., 244: 365-396).

Other examples of poxviruses that are appropriate for the purposes of the invention include duck pox, fowl pox, cow pox, entomopox, monkey pox, swine pox and penguin pox.

The poxvirus is found in two morphologically distinct forms, called intracellular mature virus (IMV) and enveloped extracellular virus (EEV).

The poxvirus used as an expression vector of the invention has at least one of the following characteristics, taken alone or in combination:

1. The poxvirus is an MVA virus,
2. The poxvirus is in the IMV morphological form, and
3. The genome of the poxvirus is modified so as to insert the two expression cassettes.

Preferably, the promoters used in poxvirus vectors as expression vectors are homologous promoters (e.g. from poxvirus origin). Representative examples include without limitation the vaccinia promoters 7.5K, H5R, TK, p28, p11 and K1L, chimeric promoters between early and late poxviral promoters as well as synthetic promoters such as those described in Chakrabarti et al. (1997, Biotechniques 23, 1094-1097), Hammond et al. (1997, J. Virological Methods 66, 135-138) and Kumar and Boyle (1990, Virology 179, 151-158). When the genome of the poxvirus is modified so as to insert the two cassettes of interest, the means necessary to their expression are both homologous. Thus, in the case where the MVA virus is used, the expression of NS3/NS4 can be for example under the control of the promoter ph5r so that the corresponding expression cassette is p7.5-NS5b, and vice versa. The expression cassettes can be inserted at the same or at different location in the poxvirus genome. In a preferred embodiment, the expression cassettes are both inserted in deletion II or in deletion III of a MVA genome, with a special preference for deletion III.

According to a particular embodiment, when the genome of the poxvirus is modified so as to insert the two cassettes of interest, the two said expression cassettes are oriented in the same direction.

According to another particular embodiment, they are oriented in the opposite direction.

Here too, the expression cassettes are inserted into the genome of the poxvirus in a manner known to a person skilled in the art, as indicated previously.

The vectors of the invention can also comprise sequences necessary for targeting peptides towards particular cell compartments. An example of targeting can be the targeting towards the endoplasmic reticulum obtained using address sequences of the leader sequence type originating from the protein E3 of the adenovirus (Cierikl I. F., et al., The Journal of Immunology, 1999, 162, 3915-3925).

They can also comprise sequences necessary for targeting towards the dendritic cells and for targeting at the membrane of the cells.

An object of the invention is also the microorganisms and the eukaryotic cells transformed by an expression vector of the invention.

By way of examples of microorganisms that are suitable for the purposes of the invention, there can be mentioned the yeasts, such as those of the following families: Saccharomyces, Schizosaccharomyces, Kluyveromyces, Pichia, Hansenula, Yarrowia, Schwammonye, Zygosaccharomyces, Saccharomyces cerevisiae, Saccharomyces pombe Saccharomyces carlsbergensis, Pichia pastoris and Kluyveromyces lactis being preferred; and the bacteria, such as E. coli and those of the following families: Lactobacillus, Lactococcus, Salmonella, Streptococcus, Bacillus and Streptomycyes.

By way of examples of eukaryotic cells, there can be mentioned cells originating from animals such as mammals, reptiles, insects and equivalent. They can be of a unique type of cells or a group of different types of cells and encompass cultured cell lines, primary cells and proliferative cells. The preferred eukaryotic cells are cells originating from the Chinese hamster (CHO cells), monkey (COS and Vero cells), baby hamster kidney (BHK cells), pig kidney (PK 15 cells) and rabbit kidney (RK13 cells), human osteosarcoma cell lines (143 B), HeLa human cell lines and the human hepatoma cell lines (Hep G2-type cells), as well as insect cell lines (for example of Spodoptera frugiperda).

The host cells can be provided in cultures in suspension or in flasks, in tissue cultures, organ cultures and equivalent. The host cells can also be transgenic animals.

Advantageously, the NS3/NS4 and/or the NS5b-encoding nucleotide sequence(s) can independently be optimized for providing high level expression in a particular host cell, e.g. mammalian, yeast or bacterial host cells. It has been indeed observed that, when more than one codon is
available to code for a given amino acid, the codon usage patterns of organisms are highly non-random (see for example Wada et al., 1992, Nucleic Acids Res. 20, 2111-2118) and may be markedly different between different hosts (see for example Nakamura et al., 1996, Nucleic Acids Res. 24, 214-215). Thus, nucleotide sequences of viral origin (HCV) may have an inappropriate codon usage pattern for efficient expression in host cells, especially bacterial or yeast cells. Typically, codon optimisation is performed by replacing one or more “native” (e.g. HCV) codon corresponding to a codon infrequently used in this particular host cell by one or more codon encoding the same amino acid which is more frequently used. This can be achieved by conventional mutagenesis or by chemical synthetic techniques (e.g. resulting in a synthetic nucleic acid molecule). It is not necessary to replace all native codons corresponding to infrequently used codons since increased expression can be achieved even with partial replacement. Moreover, some deviations from strict adherence to optimised codon usage may be made to accommodate the introduction of restriction site(s) into the resulting nucleotide sequence.

[0094] Further to optimization of the codon usage, expression in the host cell can further be improved through additional modifications. For example, the NS3/NS4 and/or NS5b-encoding nucleotide sequence can be modified so as to prevent clustering of rare, non-optimal codons being present in concentrated areas and/or to suppress or modify at least partially negative sequence elements which are expected to negatively influence expression levels (e.g. AT-rich or GC-rich sequence stretches; RNA secondary structures; and/or internal cryptic regulatory elements such as internal TATA-boxes, chi-sites, ribosome entry sites, and/or splicing donor/acceptor sites).

[0095] The invention also relates to antibodies directed against one of the peptide compositions of the invention as defined previously or against one of the expression vectors of the invention as defined previously.

[0096] The antibodies according to the invention are either polyclonal or monoclonal antibodies.

[0097] The abovementioned polyclonal antibodies can be obtained by immunization of an animal with the peptide composition of the invention or with the vector of the invention as “antigen of interest”, followed by the recovery of the antibodies sought in purified form, by sampling the serum of said animal, and separation of said antibodies from the other constituents of the serum, in particular by affinity chromatography on a column to which is fixed an antigen specifically recognized by the antibodies, in particular a viral antigen of interest.

[0098] The monoclonal antibodies can be obtained by the hybridomas technique the general principle of which is recalled hereafter.

[0099] In a first step, an animal, generally a mouse, (or cells in culture within the framework of in vitro immunizations) is immunized with the peptide composition of the invention or with the vector of the invention as “antigen of interest”, the B lymphocytes of which are then capable of producing antibodies against said antigen. These antibody-producing lymphocytes are then fused with “immortal” myelomatous cells (murine in the example) in order to produce hybridomas. From the thus-obtained heterogeneous mixture of cells, a selection is then made of cells capable of producing a particular antibody and multiplying indefinitely. Each hybridoma is multiplied in clone form, each leading to the production of a monoclonal antibody the recognition properties of which vis-à-vis the antigen of interest can be tested for example by ELISA, by immunotransfer in one or two dimensions, by immunofluorescence, or using a biocaptor. The monoclonal antibodies thus selected are subsequently purified in particular according to the affinity chromatography technique described above.

[0100] The peptide compositions, the expression vectors, the nucleotide sequences coding for said polypeptide NS3/NS4 and said polypeptide NS5b, as well as the antibodies of the invention are particularly effective for the inhibition, prevention and control of the infection of patients carrying the HCV virus, so that their use for the preparation of a medicament constitutes another object of the invention.

[0101] The present invention also relates to a pharmaceutical composition, in particular a vaccine, containing as active ingredient the peptide composition of the invention, or an expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polypeptide NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, or the nucleotide sequences coding for said polypeptide NS3/NS4 and said polypeptide NS5b, said nucleotide sequences corresponding to the sequences contained in the expression vectors of the invention, placed under the control of elements necessary to an expression constitutive of and/or inducible from said peptides, or at least one of the antibodies of the invention.

[0102] By elements necessary to an expression constitutive of the peptides, is meant a promoter that is ubiquitous or specific to the eukaryotic cells.

[0103] As elements necessary to an expression inducible from the peptides, there can be mentioned the elements of regulation of the operon of E. coli for tetracycline resistance (Gossen M. et al., Proc Natl Acad Sci USA, 89: 5547-5551 (1992)).

[0104] According to a particular embodiment of the invention, the pharmaceutical composition also contains a pharmaceutically appropriate vehicle. Of course, a person skilled in the art will easily determine the nature of the pharmaceutically appropriate vehicle and the quantity of active ingredient to be used as a function of the constituents of the pharmaceutical composition.

[0105] The quantity and nature of the pharmaceutically appropriate vehicle can be easily determined by a person skilled in the art. They are chosen according to the desired pharmaceutical form and method of administration.

[0106] In addition, the pharmaceutical composition of the invention may comprise one or more adjuvant(s) suitable for systemic or mucosal application in humans. Preferably, the adjuvant is capable of stimulating immunity against an HCV protein or one epitope, especially a T cell-mediated immunity. Representative examples of suitable adjuvants, especially for use in combination with the peptide composition of the invention, include without limitation alum, mineral oil emulsion such as Freund’s complete and incomplete (IFA), lipopolysaccharide or a derivative thereof (Kühn et al., 1986,
preferably contain as active ingredient one of the vectors of the invention or an expression vector comprising a nucleotide sequence coding for the polypeptide NS5b, so that they are useful in prophylactic and therapeutic vaccination.

Prophylactic and therapeutic vaccination can be implemented by injection of a vaccine based on one or more expression vectors of the invention, to the extent that the expression vector or vectors finally code for the polypeptide NS3/NS4 and for the polypeptide NS5b as active ingredient, said injection being or being not followed by boosters. It can also be implemented by injecting one or more doses of two different types of expression vectors of the invention, firstly an adenosivirus to prime the host's immune response, then a poxvirus to boost the primed immune response, simultaneously or at different times, and vice versa (e.g. a poxvirus vector as a primer and an adenosivirus vector as a booster).

These vectors can be contained in a pharmaceutical kit.

Also, another object of the invention is pharmaceutical kits, in particular vaccinal, comprising at least one expression vector comprising a nucleotide sequence coding for the polypeptide NS3/NS4 and at least one expression vector comprising a nucleotide sequence coding for the poxvirus type as defined previously and/or at least one expression vector of poxvirus type as defined previously.

Prophylactic and therapeutic vaccination can also be implemented by injection of a vaccine based on at least one expression vector of the invention, or an expression vector comprising a nucleotide sequence coding for the polypeptide NS3/NS4 with an expression vector comprising a nucleotide sequence coding for the poxvirus type as defined previously and/or one or more different types of expression vectors of the invention, firstly an adenosivirus to prime the host's immune response, then a poxvirus to boost the primed immune response, simultaneously or at different times, and vice versa (e.g. a poxvirus vector as a primer and an adenosivirus vector as a booster).

These vectors can be contained in a pharmaceutical kit.

Also, another object of the invention is pharmaceutical kits, in particular vaccinal, comprising at least one expression vector of the invention or at least one pharmaceutical composition of the invention or an expression vector comprising a nucleotide sequence coding for the poxvirus type as defined previously and/or at least one expression vector of poxvirus type as defined previously.
of the invention) or any combination thereof. The present invention also provides the use of at least one of the above-described active ingredient or any combination thereof for the preparation of a medicament for treating one or more pathologies associated with a hepatitis C virus. The term “treatment” or “treating” as used herein encompasses prophylactic and therapeutic vaccination of a host organism infected with a hepatitis C virus. The term “host organism” is intended to encompass any mammal, such as any murine, rat, bovine, porcine, canine, feline, equine, monkey or human subject, for example a human infected with HCV.

[0120] The method or use of the present invention is especially useful for treating HCV persistent infection and liver cancer in HCV-infected patients. The term “cancer” encompasses any cancerous conditions including diffuse or localized tumors, metastasis, cancerous polyps as well as preneoplastic lesions (e.g. cirrhosis). Desirably, the effective dose of the peptide composition, vector, pharmaceutical composition and/or antibodies of the invention is such that it provides a therapeutic benefit to the host organism into which it is administrated. The therapeutic benefit can be evidenced by a number of ways, for instance a decrease of HCV viremia detected in blood, plasma or sera of the treated organism as compared to before treatment, and/or by the detection of an anti-HCV immune response (e.g. production of anti-HCV antibodies and/or T cell-mediated immunity) or by the delay of the symptoms associated with an HCV infection (e.g. delay in the development of liver cirrhosis or cancer), or by a decrease or slow down of liver inflammation/steatosis/fibrosis conditions typically associated with HCV infection or by an improved response of the individual to conventional therapies.

[0121] Preferably, the NS3/NS4 polyprotein and eventually the NS5b polypeptide comprised or encoded by the peptide composition, vector, and/or pharmaceutical composition of the invention originates from genotype 1b and is used according to the modality described herein for treating the pathologies associated with a genotype 1b hepatitis C virus. Alternatively, it originates from genotype 1b and is used according to the modality described herein for treating the pathologies associated with a genotype other than 1b, such as a genotype 1a, 3 or 4 hepatitis C virus with a special preference for genotype 1a.

[0122] If desired, the method or use according to the invention can be carried out in conjunction with one or more conventional therapeutic modalities (e.g. radiation, chemotherapy and/or surgery). In one embodiment, the method or use of the invention is associated to chemotherapy with one or more drugs which are conventionally used for treating or preventing HCV infections, HCV-associated pathologies. Representative examples of HCV drugs include without limitation antivirals, protease inhibitors (e.g. serine protease inhibitors such as VX950 of Vertex), polymerase inhibitors, helicase inhibitors, antifibrotics, nucleoside analogs, TLR agonists, N-glycosylation inhibitors, siRNA, antisense oligonucleotides, immune modulators, therapeutic vaccines and antitumor agents usually used in the treatment of HCV-associated hepatocarcinomas (e.g. Adriamycin or a mixture of Adriamycin lipiodol and spongol usually administered by chemoembolisation in the hepatic artery). Such HCV drugs can be provided in a single dose or, alternatively, in multiple doses according to standard protocols, dosages and regimens over several hours, days and/or weeks. Their administration may precede, be concomitant, or subsequent to the administration of the peptide composition, vector, pharmaceutical composition and/or antibody of the invention. A preferred combination includes treatment of the host organism with pegylated IFN-α2a or IFN-α2b (e.g. at a dose of 10 µg/week) eventually in combination with ribavirin (e.g. at 800 to 1200 mg/day) for 24 to 48 weeks, before, in parallel or subsequently to the method or use of the invention. The peptide composition, vector, pharmaceutical composition and/or antibody of the invention can also be administered in combination with other treatments designed to enhance immune responses, e.g. by co-administration with adjuvants or cytokines (or vectors encoding cytokines) as is well known in the art.

[0123] In another embodiment, the method or use of the invention is carried out according to an accelerated immunization schedule which comprises at least three (e.g. from 3 to 10) sequential administrations of the peptide composition, vector, pharmaceutical or composition of the invention. Preferably, the at least three sequential administrations, are independently separated by a period of time varying from 3 days to 10 days but no more than 15 days. The method or use of the invention preferably comprises three sequential administrations of the vector or pharmaceutical composition of the invention, each at approximately one week interval. Even more preferably the method or use of the invention comprises three sequential administrations by intramuscular route at approximately one week interval of a poxvirus expression vector (e.g. a NS3/NS4 and NS5B-encoding MVA) as defined above or the pharmaceutical composition comprising such a vector.

[0124] In still another embodiment, the method or use of the invention can further include at least one “recall” administration at the end of the at least three sequential administrations. The number of recall administration(s) can vary from one to 10 and the time interval between the latest of the first series of sequential administrations and the first recall administration is a matter of at least approximately 4 weeks. Advantageously, the method or use comprises three sequential administrations at approximately one week interval and one recall administration which takes place either approximately 4 weeks or 6 months after the latest of the at least three sequential administrations. Alternatively, the method or use of the invention comprises two recall administrations, the first being approximately 4 weeks and the second approximately 6 months after the latest of the first series of sequential administrations. The recall administration(s) may use the same as or a different active ingredient than the first series of sequential administrations and may use the same route or a different route of administration. According to one aspect, the recall administration(s) is/are made using the same active ingredient and by the same route as the first series of sequential administrations. A preferred method or use according to this aspect comprises three sequential administrations at approximately one week interval and one or two recall administration(s) approximately 4 weeks and/or approximately 6 months after the third sequential administration, all by intramuscular route and with a poxvirus expression vector (e.g. a NS3/NS4 and NS5B-encoding MVA) as defined above or the pharmaceutical composition comprising such a vector.

[0125] According to another aspect, the sequential administrations and the recall administration(s) can use different
active ingredients and/or different routes of administration. For example, the at least three sequential administrations may be made by intramuscular route with a poxvirus expression vector (e.g. a NS3/NS4 and NS5B-encoding MVA) or the pharmaceutical composition comprising such a vector as defined above and the recall administration(s) by subcutaneous or intramuscular route with any prior art polypeptide comprising NS3, NS4 and/or NS5B such as the polypeptide described in European patent application No EP 06 36 0014.2.

**0126** Another object of the invention provides a method of stimulating a T cell-mediated immune response against a hepatitis C virus target protein which comprises the step of administering in a host organism at least one dose of the peptide composition, vector, pharmaceutical or composition of the invention so as to stimulate a host’s T cell-mediated immune response.

**0127** The stimulated immune response is preferably a CD8+ T cell response, a CD4+ T cell response or both a CD8+ and a CD4+ T cell responses. Desirably, the T cell-mediated immune response stimulated by the method or use of the present invention permits to target at least one epitope located in a NS3, and/or a NS4 and/or a NS5B protein present in the infecting hepatitis C virus. Preferably, the T cell-mediated immune response provided by the method or use of the invention is specific for at least one HLA-A restricted epitope and in particular at least one HLA-A-B7 epitopes located in the NS3 polypeptide of the infecting hepatitis virus. Alternatively or in combination, the stimulated T cell-mediated immune response is specific for at least one HLA-A2-restricted epitope and in particular at least one HLA-A2 epitope located in the NS3 and/or NS5B protein of the infecting hepatitis virus.

**0128** In a preferred embodiment, the method or use according to the invention is provided to the host organism according to the accelerated immunization schedule described above and comprises at least three sequential administrations of the peptide composition, vector, pharmaceutical or composition of the invention and optionally one or two recall administration(s).

**0129** Desirably, the stimulated T cell immune response is long-lasting and can be detected in the treated host organism for at least one month following the last administration of the peptide composition, vector, pharmaceutical or composition of the invention. Preferably, the stimulated immune response can be detected for at least 2 months, desirably for at least 3 months, and preferably for at least 6 months.

**0130** The ability of the method or use of the invention to stimulate an anti-HCV T cell-mediated immune response can be evaluated either in vitro or in vivo using a variety of assays which are standard in the art. For a general description of techniques available to evaluate the onset and activation of an immune response, see for example Coligan et al. (1992 and 1994, Current Protocols in Immunology; ed J Wiley & Sons Inc, National Institute of Health). Measurement of cellular immunity can be performed by measurement of cytokine profiles secreted by activated effector cells including those derived from CD4+ and CD8+ T-cells (e.g. quantification of IL-10 or IFNγ-producing cells by ELISpot), by determination of the activation status of immune effector cells (e.g. T cell proliferation assays by a classical [3H] thymidine uptake), by assaying for antigen-specific T lymphocytes in a sensitized subject (e.g. peptide-specific lysis in a cytotoxicity assay). The method of the invention can also be further validated in animal models challenged with an appropriate infectious agent (e.g. a bacteria or a vaccinia virus expressing HCV genes) to determine neutralization of the infectious agent and eventually partial resistance to the associated symptoms, reflecting an induction or an enhancement of an anti-HCV cell immune response. Testing and validation of the vector compositions of the invention are also illustrated in the appended Example section.

**0131** The present invention will be better understood using the following examples that are given only by way of illustration, and are non-limitative, as well as using the attached FIGS. 1 to 16 in which:

**0132** FIG. 1A to 1K represents the maps of the different plasmids used for obtaining an adenovirus AdNS3NS4NS5b according to the invention, on which are indicated the sites of the different restriction enzymes and the location of the sequence fragments coding for NS3/NS4 and for NS5b,

**0133** FIG. 2A to 2I represents the maps of the different plasmids used for obtaining a poxvirus MVA NS3NS4NS5b according to the invention, on which are indicated the sites of the different restriction enzymes and the location of the sequence fragments coding for NS3/NS4 and for NS5b,

**0134** FIG. 3 gives the cell response induced by the adenovirus AdNS3NS4, either according to the CTL test (FIG. 3A) where the epitope GLL was used for stimulating the splenocytes in culture and for loading the CTL targets and the result of which is expressed as a specific lysis percentage as a function of the effector/target ratio, or according to the ELISPOT test (FIG. 3B), specific to the epitope GLL, where the result is given in numbers of spots/10 cells,

**0135** FIG. 4 gives the cell response induced by the adenovirus AdNS5b according to the test ELISPOT, specific to the epitopes ALY and KLQ,

**0136** FIG. 5 gives the cell response induced by the adenovirus AdCEIE2 according to the CTL test where the epitope DLM was used for stimulating the splenocytes in culture and for loading the targets of the CTL and the result of which is expressed as a specific lysis percentage as a function of the effector/target ratio,

**0137** FIG. 6 gives the titre of the recombinant vaccinia virus, resulting from the trial test, in pfu/ml/mg ovary, for the 3 groups of 8 mice immunized by the following different combinations of adenovirus: AdNS3NS4+AdNS5b (1st group), the adenoviruses AdNS3NS4+AdNS5a (2nd group), the adenoviruses AdNS3NS4+AdNS5b+AdCEIE2 (3rd group) and the adenovirus AdβGal (4th group) and

**0138** FIG. 7 gives the titre of the recombinant vaccinia virus, resulting from the trial test, in pfu/ml/mg ovary, for the 2 groups of 8 mice immunized by the following different combinations of adenovirus: AdNS3NS4NS5b (1st group), AdNS3NS4+AdNS5b (2nd group) and AdβGal (3rd group).

**0139** FIG. 8 illustrates in vitro analysis of NS3, NS4 and NS5B expression by flow cytometry following Huh-7 infection by MVA NS3/4-NS5B. MVA NS3/4-NS5B infected Huh-7 cells were harvested 24 h after infection with a MOI of 1 and stained using mouse monoclonal anti-NS3 (8D8E1), anti-NS4B (1B12A3) and anti-NS5B (5I12B7)
antibodies. Results are expressed as percentage of positive cells compared with MVA N33 (MVA wild type) infected cells. Bold lines: cells infected with MVA NS34-NS5B, thin lines: cells infected with MVA N33, shadowed histogram: non infected cells. MFI: mean fluorescence intensity.

FIG. 9 provides a comparison of IFNγ-producing CD8+ T cell frequencies specific of NS3 HLA-A2 restricted epitopes induced in HLA-A2 transgenic mice following two different immunization schedules with the MVA NS34-NS5B. Schedule 1: 3 sub-cutaneous (sc) injections performed at wk 1, 4, 7. Schedule 2: 4 sc injections performed at wk 1, 2, 3 and 6. (A) IFNγ ELISPOT assays performed at wk 6 (schedule 1) or wk 5 (schedule 2). (B) IFNγ ELISPOT assays performed at wk 9 (schedule 1) or wk 8 (schedule 2). IFNγ ELISPOT assays were performed as described in Materials and Methods. M1, M2 and M3 represent 3 mice immunized with the MVA NS34-NS5B. N33 is a representative MVA N33 injected control mouse. NS3 HLA-A2 restricted epitopes GLL and KLT or irrelevant peptide were used for restimulation. Each bar represents the response of a single immunized mouse. The dashed horizontal line represents the cut-off above which IFNγ producing T cell frequency is considered positive.

FIG. 10 illustrates cytotoxic CD8+ T cell responses specific of NS3 or NS5B HLA-A2 restricted epitopes induced in HLA-A2 transgenic mice following administration of MVA NS34-NS5B according to two different immunization schedules. Schedule 1: 3 sc injections performed at wk 1, 4, 7. Schedule 2: 4 sc injections performed at wk 1, 2, 3 and 6. (A) CTL assays performed at wk 6 (schedule 1) or wk 5 (schedule 2). (B) CTL assays performed at wk 9 (schedule 1) or wk 8 (schedule 2). CTL assays were performed as described in Materials and Methods. Data represent the % of specific lysis obtained at different effector to target cell (E/T) ratios. HLA-A2 restricted peptides used to pulse target cells are indicated on top of each graph: GLL for NS3, ALY for NS5B. Each bar represents the response of a single immunized mouse.

FIG. 11 illustrates CD8+ T cell responses characterized by ICS and CTL assays following administration in HLA-A2 transgenic mice of MVA NS34-NS5B according to the accelerated immunization schedule. (A) ICS assay. The left part of the Figure represents IFNγ+CD8+ cell dot plots representative of MVA NS34-NS5B and MVA N33 immunized animals and following gating on CD3+ CD8+ cells performed as described in Materials and Methods. GLL peptide or irrelevant peptide were used for restimulation. Histogram on the right represents percentage of IFNγ+CD8+ cells detected for 4 MVA NS34-NS5B and 2 MVA N33 immunized mice following GLL restimulation. Empty bars: response of a single immunized mouse, black bars: median values. (B) In vivo CTL assay. GLL-pulsed CFSE high and unpulsed CFSE low target cells were injected into recipient mice as described in Materials and Methods. Twenty hours later, the percentage of target cells killed was evaluated in the spleen. The percentages of specific lysis are indicated for each MVA NS34-NS5B mouse (M1 to M4).

FIG. 12 illustrates IFNγ-producing T cell responses following administration of MVA NS34-NS5B in HLA-B7 transgenic mice according to the accelerated immunization schedule. IFNγ ELISPOT assays were performed as described in Materials and Methods. M1-5 represents 5 mice immunized with the MVA NS34-NS5B and M6-7, 2 mice immunized with the MVA N33. Hatched, dot and empty bars represent values obtained for individual mouse and specific of HLA-B7-restricted WPA10, LSP10 or irrelevant peptide epitopes, respectively. Median values are presented for MVA NS34-NS5B injected mice with black (WPA10 peptide) and grey (LSP10 peptide) bars. The dashed horizontal line represents the cut-off above which IFNγ producing T cell frequency is considered positive.

FIG. 13 illustrates IFNγ+CD4+ T cell responses specific of NS3, NS4 and NS5B antigens following administration of MVA NS34-NS5B in Balb/C mice according to the accelerated immunization schedule. CD4+ T cells were positively selected from splenocytes of individual MVA NS34-NS5B or MVA N33 immunized mouse. Total splenocytes, CD4+ fraction and effluent fraction were generated and IFNγ ELISPOT assays performed on each fraction for individual mice as described in Materials and Methods. HCV NS3, NS4, NS5B recombinant antigens or TT (irrelevant stimulus) were used for restimulation. Results are shown as bars representing the median spots value detected for 106 splenocytes, obtained for groups of 4 MVA NS34-NS5B (hatched bars) or 2 MVA N33 (empty bars) immunized mice. Within bars, results obtained for each mouse are represented as black dots. The dashed horizontal line represents the cut-off above which IFNγ producing T cell frequency is considered positive.

FIG. 14 illustrates the longevity of CTL responses following administration of MVA NS34-NS5B in HLA-A2 transgenic mice according to the accelerated immunization schedule. Mice received 4 MVA NS34-NS5B injections at week 1, 2, 3 and 27 and CTL assays were performed as described in Materials and Methods at week 5, 10, 27 and 29. Four MVA NS34-NS5B and 2 MVA N33 injected mice were sacrificed for each time point analyzed. Hatched bars: median % of lysis values for MVA NS34-NS5B injected mice, white bars: median % of lysis values for MVA N33 injected mice, at different effector to target (E/T) ratios.

FIG. 15 illustrates the longevity of IFNγ ELISPOT responses in HLA-A2 transgenic mice following administration of MVA NS34-NS5B according to the accelerated immunization schedule. Mice received 4 MVA NS34-NS5B injections at week 1, 2, 3 and 27 and ELispot assays were performed as described in Materials and Methods at week 5, 10, 27 and 29. Four MVA NS34-NS5B and 2 MVA N33 injected mice were sacrificed for each time point analyzed. Hatched, white and black bars represent values obtained for individual mice for specific and an irrelevant peptide, respectively. Median values are represented for MVA NS34-NS5B injected mice with black (specific peptide) and dotted bars (irrelevant peptide). The dashed horizontal line represents the cut-off above which IFNγ production is considered positive.

FIG. 16 illustrates the residual bacterial titers in spleen and liver of MVA NS34-NS5B immunized mice challenged with TC-LNS3 bacteria. (A) Bacterial titers in vaccinated HLA-A2 transgenic mice. Four mice were immunized either with the MVA NS34-NS5B or the MVA N33 (negative control) according to the “accelerated” schedule of vaccination or with TC-LNS3 two times at 2 weeks.
interval using a low immunization dose (0.05 to 0.1 LD₅₀) (positive control), 15 days prior challenge. Challenge was performed with a dose of 1 LD₅₀ of TC-LNS3 bacteria and mice were sacrificed 2 days later. Residual bacterial titers were evaluated by titrations of livers and spleens serial dilutions. Individual mice in each group are represented by circular symbols and the median value obtained for each group is represented by the black and thick line. P values are calculated according to the non parametric test of Mann Whitney and values are considered as statistically different when p<0.05. (B) Bacterial titers in vaccinated Balb/c mice. Challenge was carried out as described in (A) except that 6 mice were included in each animal group.

**EXAMPLE 1**

**Preparation of an Adenovirus Allowing the Expression of the Proteins NS3/NS4 and NS5b According to the Invention**

**[0148]** 1. Adenovirus

**[0149]** The recombinant adenoviruses are generated by transfection (CalP30) of the complementation line 293 (Graham, Smiley, et al. 1977) after linearization of the genomes by PacI. The recombinant viruses propagate and are amplified on this same line, and their purification is carried out from the infected cells. The cells are recovered by centrifugation (1500 rpm, 10 minutes) and lysed by 3 freeze/thaw cycles. The cell lysate is clarified by two centrifugations (2000 rpm, 10 minutes; 8000 rpm, 15 minutes), then purified by two successive ultracentrifugations. The first is carried out on a Caesium Chloride gradient (densities 1.4 and 1.25) at 30,000 rpm for 1 hour. The second is carried out on a Caesium Chloride cushion (density 1.34) at 35,000 rpm for 18 hours. The phases containing the virions are removed and diluted by half in a 60% saccharose buffer. The viral suspensions are then dialysed against formulation buffer (for 10 litres: 3423 g of saccharose; 12.11 g of Tris; 2.033 g of MgCl₂; 87.7 g of NaCl), then aliquoted. Their titration is carried out by indirect immunofluorescence on 293 cells infected by different viral dilutions and marked by an antibody specific to the adenoviral DNA-Binding Protein (a72K B6-8) (Reich, Sarnow, et al. 1983).

**[0150]** 2. Preparation of the Adenovirus AdNS3NS4

**[0151]** This adenovirus allows the expression of the gene coding for the polyprotein NS3/NS4 (SEQ ID NOs: 1 and 2) under the control of the CMV promoter.

**[0152]** 2.1 PCR Amplification of the Nucleotide Sequence Coding for the Polyprotein NS3/NS4

**[0153]** In order to do this, the following oligonucleotides were used:

-oIV166: (SEQ ID NO: 9)

5'-GGG GGG GCT ATG GCC CCT ATC ACG GCC TA-3'

-oIV171: (SEQ ID NO: 10)

5'-GGG GGG GCT TTA GCA TGA CTT GGA GCA GT-3'

**[0154]** as well as the following reagents:

Taq DNA Polymerase, PCR buffer, MgCl₂, 1.5 mM and dNTP 10 mM (Invitrogen).

**[0155]** The PCR conditions were the following:

[0156] 5 minutes at 94° C., then

[0157] 30 cycles of the series: 45 seconds at 94° C., 45 seconds at 62° C. and 1 minute at 72° C., then

[0158] 10 minutes at 72° C.

[0159] 2.2 Insertion of the PCR Fragment NS3/NS4 into the Transfer Plasmid pTG13387

**[0160]** The following stages were carried out:

**[0161]** Enzymatic digestion of the plasmid pTG13387 (FIG. 1A, Transgene) by Nhel/MluI (Nhel, Invitrogen in React 4 Buffer and MluI, Invitrogen in React 3 Buffer)

**[0162]** Enzymatic digestion of the fragment NS3/NS4 by Nhel/MluI

**[0163]** Ligation (T4 DNA Ligase (Invitrogen)) in Reaction Buffer (Invitrogen)),

**[0164]** Bacterial transformation (strain 5K, (Transgene)

**[0165]** Selection of bacterial clones on LB medium (Difco)ampicillin (100 µg/ml, Duchefa)

**[0166]** Plasmid maxi-preparation (Qiagen, according to manufacturer’s protocol) of a positive clone after restriction analysis

**[0167]** Restriction analysis: digestion by Smal (Invitrogen in React 4 Buffer) and obtaining of fragments of: 5450, 2164, 909, 214 and 180 pb

**[0168]** Obtaining of the plasmid pIV315 deleted from its E1 region and containing the sequence NS3/NS4 under the control of the CMV promoter (FIG. 1B).

**[0169]** 2.3 Homologous Recombination with the Complete Adenoviral Genome Deleted from its E3 Region Contained in the Plasmid pTG6624

**[0170]** The following stages were carried out:

**[0171]** Enzymatic digestion of the plasmid pIV315 obtained above by PacI/PvuI (PacI in NEB1 buffer, Biolabs and PvuI in React 7 Buffer, Invitrogen); isolation on agarose gel of the fragment containing the cassette pCMV-NS3-NS4

**[0172]** Enzymatic digestion of the plasmid pTG6624 (FIG. 1C) by ClaI (in React 1 Buffer, Invitrogen)

**[0173]** Bacterial transformation (strain BJ, (Transgene) in order to carry out the homologous recombination between the two plasmid fragments

**[0174]** Selection of bacterial clones on LB medium-ampicillin (100 µg/ml)

**[0175]** Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis

**[0176]** Restriction analysis: digestion by Smal and obtaining of fragments of: 2263, 621, 3814, 214, 2164, 909, 180, 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230 and 3685 pb

**[0177]** Obtaining of the complete adenoviral genome Adenovirus AdNS3NS4, deleted from its E3 and E1 regions, the latter having been replaced by the expression cassette pCMV-NS3-NS4 (pIV317, FIG. 1D).
3. Preparation of the Adenovirus AdNS3NS4NS5b

This adenovirus allows the expression of the gene coding for the polyprotein NS3/NS4 under the control of the CMV promoter and the expression of the gene coding for the polypeptide NS5b under the control of the SV40 promoter.

3.1 Construction of the Transfer Plasmid Allowing the Cloning in the E3 Region of the Adenovirus of a Coding Sequence Under the Control of the CMV Promoter

The following stages were implemented:

- Enzymatic digestion of the plasmid pTG4664 (FIG. 1E, Transgene) by BglII (in React 3 Buffer, Invitrogen)
- Enzymatic digestion of the plasmid pTG3074 (FIG. 1F, Transgene) by BamHI/BglII (in React 3 Buffer, Invitrogen)
- Ligation (T4 DNA ligase), bacterial transformation (strain 5K)
- Selection of bacterial clones on LB medium+ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by SmaI and obtaining of fragments of: 4940, 1305 and 230 pb
- Obtaining of the plasmid pIV267 (FIG. 1G)
- Digestion of the plasmid pIV267 thus obtained by ClaI/MunI (in React 1 Buffer, Invitrogen)
- Treatment by DNA Polymerase I, Large (Klenow) Fragment (in React 2 Buffer, Invitrogen)
- Ligation (T4 DNA Ligase), bacterial transformation (strain 5K)
- Selection of the bacterial clones on LB medium+ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by SmaI and obtaining of fragments of: 4692, 1305 and 230 pb

3.2 Replacement of the CMV Promoter by the SV40 Promoter in pIV270

The following stages were carried out:

- PCR amplification of the nucleotide fragment corresponding to the SV40 promoter, from the commercial plasmid pcDNAHygro (Clonetech) using the following oligonucleotides:
  - oIV232:
    ```
    5'-GGG GGG AGA TCT CCA GCA AGA AGT ATG-3'  
    ```
  - oIV233:
    ```
    5'-GGG GGG GTC GAC CGA AAA TGG ATA TAC AAG CTC-3'  
    ```

and according to the procedure described in point 2.1 above, except that a temperature of 58°C instead of 62°C was used

- Enzymatic digestion of pIV270 by BglII/Sall (in React 10 Buffer, Invitrogen)
- Enzymatic digestion of the PCR fragment by BglII/ Sall
- Ligation (T4 DNA ligase), bacterial transformation (strain 5K)
- Selection of the bacterial clones on LB medium+ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by SmaI and obtaining of fragments of: 4692, 719, 80 and 230 pb
- Obtaining of the plasmid pIV330, transfer plasmid allowing the cloning in the E3 region of the adenovirus of a coding sequence under the control of the SV40 promoter (FIG. 11)

3.3 Insertion of the PCR Fragment NS5b into the Transfer Plasmid pIV330

The following stages were carried out:

- PCR amplification of the nucleotide sequence coding for the protein NS5b (SEQ ID NOs: 3 and 4) using the following nucleotides:
  - oIV212:
    ```
    5'-GGG GGG TCT AGA ATG TCA ATG TCC TAC ACA TGG AC-3'  
    ```
  - oIV21s:
    ```
    5'-GGG GGG TCT AGA TTA CCG GTT GGG GAG CAG GT-3'  
    ```

and according to the procedure described in point 2.1 above, except that a temperature of 60°C instead of 62°C was used

- Enzymatic digestion of the plasmid pIV330 obtained above by Xbal (in React 2 Buffer, Invitrogen)
- Enzymatic digestion of the PCR fragment by Xbal
- Ligation (T4 DNA Ligase), bacterial transformation (strain 5K)
- Selection of the bacterial clones on LB medium+ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis
- Restriction analysis: digestion by SmaI and obtaining of fragments of: 4692, 1505, 760, 719 and 230 pb
- Obtaining of the plasmid pIV336, transfer plasmid in the E3 deletion containing the sequence NS5b under the control of the SV40 promoter (FIG. 1J)

3.4 Homologous Recombination with the Recombinant Adenoviral Genome pIV317 in Order to Obtain the Adenovirus of the Title
The following stages were implemented:

Digestion of the plasmid pIV317 obtained in point 2.3 above by SfI (in Universal Buffer, Stratagene)

Digestion of the plasmid pIV336 obtained in point 3.3 by NheI/SaclI (in Buffer T, Amersham Pharmacia Biotech) and isolation on agarose gel of the fragment containing the cassette pSV40-NS5b

Bacterial transformation (strain BJ) for carrying out the homologous recombination between the two plasmid fragments

Selection of the bacterial clones on ampicillin (100 µg/ml)

Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis

Restriction analysis: digestion by Smal and obtaining of fragments of: 6480, 4456, 3814, 3540, 3386, 2739, 2463, 2263, 2164, 1455, 1105, 909, 760, 719, 621, 230, 214 and 180 pb

Obtaining of the desired complete adenoviral genome, deleted from the El region, the latter having been replaced by the expression cassette pCMV-NS3-NS4, and deleted from the E3 region, the latter having been replaced by the expression cassette pSV40-NS5b (plasmid pIV342, FIG. 1K).

Confirmation of the Expression of the Antigens Inserted into the Different Adenoviruses

The expression of the HCV antigens encoded by the adenoviruses AdNS3NS4, AdNS5b and AdNS3NS4NS5b was verified by Western blot after infection of Huh? cells.

As expected, all the antigens were expressed.

EXAMPLE 2
Preparation of a Poxvirus Allowing the Expression of the Proteins NS3/NS4 and NS5b According to the Invention

1. MVA Poxvirus

The strain Modified Virus Ankara MVATG N33 was supplied by TRANSGENE S. A. (Strasbourg, France).

2. Preparation of the Transfer Plasmid Allowing the Expression of the Gene NS3/NS4 Under the Control of the ph5r Promoter

2.1 Construction of the pIV250 Vector Containing the Recombination Arms BRG2 and BRD2 of the MVA, as well as the Selection Gene GPT Under the Control of the Promoter ph5r (MVA), Followed by a Second Promoter ph5r in Order to Allow the Expression of the Gene of Interest

At this point, the insertion of the fragment ph5r-GPT-BRG3-ph5r (originating from the plasmid pTG9997, Transgene) into the plasmid pTG6018 (Transgene) containing the recombination arms BRG2 and BRD2 is desired.

In order to do this, the following stages were carried out:

Enzymatic digestion by BamH1, then partial digestion by ScaI of the plasmid pTG9997 (FIG. 2B)

Purification according to the QIAGEN protocol of the restriction fragment of 1047 pb that contains the sequence coding for ph5r-GPT-BRG3-ph5r

Ligation (T4 DNA Ligase), bacterial transformation (strain TG1, Statagene)

Selection of the bacterial clones on ampicillin (100 µg/ml)

Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis (EcoRV+HindIII (in React 2 Buffer, Invitrogen): fragments of 246, 439, 476, 826 and 2789 pb; ScaI: fragments of 915 and 3861 pb)

Obtaining of the plasmid aimed at (pIV250, FIG. 2C).

2.2 PCR Amplification of the Nucleotide Sequence Coding for the Polypeptide NS3/NS4

The following oligonucleotides were used:

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52° C. instead of 62° C. was used.

2.3 Insertion of the Fragment of PCR NS3-NS4 in the Plasmid pIV250

In order to do this, the following stages were carried out:

Enzymatic digestion of the plasmid pIV250 obtained in point 2.1 above by PstI (in React 2 Buffer, Invitrogen)/XbaI

Enzymatic digestion of the PCR fragment NS3/NS4 by PstI/XbaI

Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)

Selection of the bacterial clones on ampicillin (100 µg/ml)

Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis: (HindIII (in React 2 Buffer, Invitrogen): fragments of 4763 and 2789 pb; SphI (in React 6 Buffer, Invitrogen): 1534 and 5991 pb; NcoI (in React 3 Buffer, Invitrogen): 2764 and 4761 pb)

Obtaining of the transfer plasmid containing the sequence coding for the polypeptide NS3/NS4 under the control of the promoter ph5r (pIV327, FIG. 2D).

3. Preparation of the Plasmid pIV328 Allowing the Expression of the Protein NS5b Under the Control of the p7.5 Promoter
3.1 PCR Amplification of the Nucleotide Sequence Coding for the Protein NS5b

The following nucleotides were used:

- oIV227: (SEQ ID NO: 17)
  5'-GGG GGG GTC GAC ATG TCA ATG TCC TAC ACA TGG AC-3'

- oIV228: (SEQ ID NO: 18)
  5'-GGG GGG GCA TGC TTA CCG GTT GGG GAG CAG GT-3'

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52° C. instead of 62° C. was used.

3.2 Obtaining of the Plasmid

The following stages were carried out:

- Enzymatic digestion of the PCR fragment coding for NS5b by Sall/Sphl
- Enzymatic digestion of pTG186 (FIG. 2E, Transgene) by Sall/Sphl
- Dephosphorylation of the vector pTG186 (ROCHE alkaline phosphatase)
- Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)
- Selection of the bacterial clones on ampicillin (100 µg/ml)
- Plasmid maxi-preparation (Qiagen) of a positive clone after restriction analysis: (HindIIl: fragments of 1984, 2627 and 4437 pb; BglII: fragments of 321, 557, 1361, 1451, 2237 and 3121 pb; KpnI (in React 4 Buffer, Invitrogen): fragments of: 2787 and 6261 pb)

Obtaining of the transfer plasmid containing the sequence coding for the polypeptide NS5b under the control of the p7.5 promoter (pIV328, FIG. 2F).

4. Preparation of the Transfer Plasmids pIV329 and pIV344 Allowing the Expression of the Gene Coding for the Polyprotein NS3/NS4 Under the Control of the ph5r Promoter and of the Protein NS5b Under the Control of the p7.5 Promoter

In order to do this the following stages were implemented:

- PCR amplification of the nucleotide sequence coding for the protein NS5b from the plasmid pIV328 obtained in point 3.2 above using the following oligonucleotides:
  - oIV229: (SEQ ID NO: 19)
    5'-GGG GGG TCT AGA CCG GTA GTT CGC ATA TAC ATA-3'
  - oIV218: (SEQ ID NO: 14)
    5'-GGG GGG TCT AGA TTA CCG GTT GGG GAG CAG GT-3'

and according to the procedure described in Example 1, point 2.1 above, except that a temperature of 52° C. instead of 62° C. was used.

Enzymatic digestion of the fragment of PCR by XbaI

Enzymatic digestion of the plasmid pIV327 obtained in point 2.3 above by XbaI

Ligation (T4 DNA Ligase), bacterial transformation (strain TG1)

Selection of the bacterial clones on ampicillin (100 µg/ml)


Obtaining either of the transfer plasmid allowing the expression of the polyprotein NS3/NS4 under the control of the ph5r promoter and of the protein NS5b under the control of the p7.5 promoter, the 2 expression cassettes being oriented in the same direction (pIV329, FIG. 2G), or of the transfer plasmid allowing the expression of the polyprotein NS3/NS4 under the control of the ph5r promoter and of the protein NS5b under the control of the p7.5 promoter, the 2 expression cassettes being oriented in opposite directions (pIV344, FIG. 2H).

5. Confirmation of the Expression of the Antigens Inserted into the Different Poxviruses

It was verified by Western blot, after infection of Huh7 cells with the poxviruses concerned, that the poxviruses pIV329 and pIV344, containing the sequences coding for the polyprotein NS3/NS4 and the polypeptide NS5b, expressed said HCV antigens.

EXAMPLE 3

Demonstration of the Immunogenicity of the Combination of NS3/NS4 and NS5b

1. Immunization of Mice

HLA-A2.1 transgenic mice were immunized, once, by intramuscular injection of at least one adenovirus chosen from the following adenoviruses:

- AdNS3NS4 prepared in Example 1 above (point 2.3),
- AdNS5 prepared in Example 1 above (point 3.3),
- AdNS5a prepare according to the procedure of Example 1, point 2, except that the following nucleotide primers were used in order to amplify the nucleotide sequence coding for the polypeptide NS5a (SEQ ID NOs: 5 and 6):
  - oIV172: (SEQ ID NO: 20)
    5'-GGG GGG GGT ACC ATG TCC GGC TCG TGG CTA AGG-3'
  - oIV173: (SEQ ID NO: 21)
    5'-GGG GGG GCT AGA TTA GCA GCA GAC GAT GTC GTC-3'
in the PCR the temperature of 62°C was replaced by 56°C, the enzymatic digestion of pTG 13387 and of the fragment NS5a were implemented by Kpnl/Xbal, restriction analysis by digestion by Smal of pTG13387 producing fragments of 180 and 7251 pb and of pTG6624 producing fragments of 2263, 621, 5615, 180, 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230 and 3685 pb.

[0280] AdCE1E2 according to the procedure of Example 1, point 2, except that the following nucleotide primers were used in order to amplify the nucleotide sequence coding for the core-E1-E2 polyprotein (also called CE1CE2) (SEQ ID NOs: 7 and 8):

\[
\text{oIV62:} \quad \text{(SEQ ID NO: 22)}
\]
\[5'-GGG GGG GCT AGC ATT ACC ACA AAT CCT AAA CCT-3',\]

\[
oIV69: \quad \text{(SEQ ID NO: 23)}
\]
\[5'-GGG GGG GCT AGC ATG AGA ACA AAT CCT AAA CCT-3',\]

in the PCR the temperature of 62°C was replaced by 56°C, the enzymatic digestion of pTG13387 and of the fragment CE1E2 were implemented by Nhel/Xbal, restriction analysis by digestion by Smal of pTG13387 producing fragments of 165, 435, 2270, 180 and 5254 pb and of pTG6624 producing fragments of 2263, 621, 3615, 180, 2463, 6480, 1398, 4456, 1455, 3540, 3386, 230 and 3685 pb.

[0281] AdNS3NS4NS5b prepared in Example 1 above (point 3) and

[0282] AdβGal (Transgene), according to the following protocol:

[0283] 10⁹ pfu of AdNS3NS4 or

[0284] 10⁹ pfu of AdNS5b or

[0285] 10⁹ pfu of AdCE1E2 or

[0286] 10⁹ pfu of AdNS3NS4 and 10⁹ pfu of AdNS5b or

[0287] 10⁹ pfu of AdNS3NS4, 10⁹ pfu of AdNS5b and 10⁹ pfu of AdNS5a

[0288] 10⁹ pfu of AdNS3NS4, 10⁹ pfu of AdNS5b and 10⁹ pfu of AdCE1E2

[0289] 10⁹ pfu of AdNS3NS4 NS5b or

[0290] 10⁹ pfu of Adβ-Gal as control.

[0291] Before immunization, the expression of the HCV and β-Gal antigens by the different adenoviruses used for the immunization was verified by Western blot.

[0292] 2. CTL and ELISpot Tests

[0293] Fifteen days after injection, the cell response was analyzed by isolating the spleen cells (splenocytes) of the mice and a CTL test and an ELISpot test were carried out as follows:

[0294] For the CTL test, these splenocytes were cultured on 24-well plates in the presence of:

[0295] 5 µM of the epitope GLL (GLLGYGGLL, SEQ ID NO: 24) in the case of the splenocytes originating from mice having received AdNS3NS4, 5 µM of the epitope ALY (ALYDVVSTL, SEQ ID NO: 25) or 5 µM of the epitope K1Q (K1QDCTMLV, SEQ ID NO: 26) in the case of the splenocytes originating from mice having received AdCE1E2, said epitopes being in synthetic peptide form (Eurogentex) and:

[0296] 10 U of murine recombinant interleukin 2 (Brinster et al., Heuplology 2001) per ml in alpha minimum essential medium (αMEM) for 5 days. On the 5th day, the restimulation stage was carried out, which consists of adding naive mice splenocytes to the splenocytes in culture in the presence of said epitopes over 2 days. On the 7th day, the CTL test was carried out, which consists of bringing into contact the splenocytes from the immunized mice after 7 days of culture (effector cells) and EL4 S3-Rob HDD cells loaded with 10 µM of said epitopes and labelled with Cr⁵¹ (target cells). The specific cytotoxic activity of the effector cells was determined by measuring, after 4 hours of incubation with the target cells, Cr⁵¹ released following lysis of the target cells using a γ-Cobain II counting apparatus (Packard, Rungis, France) The maximum spontaneous release from wells containing either medium alone, or lysis buffer (HCl IN) was determined. The specific percentage of cytotoxicity was calculated by the formula: (release in the test-spontaneous release)/(maximum release-spontaneous release) x 100. The epitope-specific lysis was determined by the difference between the percentage of specific lysis obtained in the presence or in the absence of said epitopes.

[0297] The ELISpot test was carried out by culturing the splenocytes for 48 hours in Multiscreen 96-well plates (Millipore) previously coated with anti-interferon gamma antibodies (IFNy) (10 µg/ml final). The splenocytes were cultured in the presence of 10 µM of the appropriate epitopes, as indicated above, and of 10 U of murine recombinant interleukin 2 per ml in αMEM. For the positive control, the splenocytes were cultured in the presence of concanavalin A (5 µg/ml). For the negative control, the splenocytes were cultured either in the absence of a non-specific peptide belonging to the capsid protein of HCV, of sequence DLGYIPPEV (also called irrelevant peptide), or in medium alone without epitope. The wells were washed three times, with 0.05% PBS-Tween then PBS respectively, an operation followed by incubation for 2 hours with anti-IFNy antibodies from biotinylated mice. After washing, the wells were incubated for 1 hour with a streptavidine-horse-radish peroxidase conjugate and the enzymatic activity was developed by degradation of the AEC (aminoethylcarbazole) substrate. The spots obtained were counted using a Zeiss ELISpot reader (Zeiss microscope in conjunction with the KS-ELISpot software).

[0298] The results are indicated in FIGS. 3 to 5 in which M corresponds to mouse and Mneg corresponds to the control mouse.

[0299] These results demonstrate that

[0300] AdNS3NS4 clearly induces a cell-mediated response specific of the expressed antigens, as illustrated in FIGS. 3A and 3B by the detection of T lymphocytes specific to the epitope GLL contained in NS3.

[0301] AdNS5b clearly induces a cell-mediated response specific of the expressed antigens, as illustrated in FIG. 4 by
the detection of T lymphocytes specific to the epitope ALY and KLQ contained in NS5b.

[0302] AdCEIE2 clearly induces a cell-mediated response specific to the expressed antigens, as illustrated in FIG. 5 by the detection of T lymphocytes specific to the epitope DLM contained in the Core protein.

[0303] 3. In Vivo Trial Test Using a Recombinant Vaccinia Virus

[0304] In order to evaluate whether the specific immune responses induced by the different adenoviruses were capable of inducing protection against an infectious disease trial ("in vivo protection"), we subjected the vaccinated mice to such a trial.

[0305] The mice not being directly infectable by HCV, in order to link the induction of a specific immune response and resistance to an infection, we used a recombinant vaccinia virus (strain WR) coding for the non-structural proteins of HCV (NS2 to NS5b) in order to carry out this trial. This recombinant vaccinia virus, after intra-peritoneal injection of 10^7 PFU in the mouse, will be replicated in the animal. The replication of this virus induces an immune response both specific to the vaccinia antigens and specific to the HCV antigens, as it also expresses the NS proteins of HCV. This specific response to the HCV antigens will be all the more effective and vigorous as the mice will have already received a vaccine expressing the HCV antigens. In other words, the more the effective vaccination (in the present case carried out with the recombinant adenoviruses) has been (i.e. the immune system of the mice have been effectively "primed" by the vaccine), the stronger will be the anti-HCV response generated after trial by the recombinant vaccinia virus and, consequently, the more the mice are "protected" against this trial. In practice, the lower the residual vaccinia virus count in the mice, the more effective the protection or the neutralization due to the vaccination has been.

[0306] The neutralization of the vaccinia virus reflects both the cell response induced by the HCV proteins and by the vaccinia proteins. The neutralization is evaluated by titration of the residual vaccinia virus from the ovaries of the animals as follows: the ovaries are removed 4 days post-trial, sonicated, freeze-thawed 3 times then after centrifugation, successive dilutions of supernatant are titrated according to the lysis plaque technique (Murata et al., PNAS, vol. 100, p. 6753-6758) on Huh7 cells. The viral titres are determined in PFU/ml/mg of ovary.


[0308] The recombinant virus titre of the vaccine was determined for 4 groups of 8 mice immunized by the following combinations of adenoviruses: AdNS3NS4+AdNS5b (1st group), AdNS3NS4+AdNS5b+AdNS5a (2nd group), AdNS3NS4+AdNS5b+AdCE1E2 (3rd group) and AdβGal (4th group).

[0309] The results, given in FIG. 6, are treated statistically on the basis of the Wilcoxon Mann-Whitney non-parametric test (Méthodes Statistiques à l’usage des médecins et des biologistes, Collection Statistique en Biologie et en Médecine, Flammarion Medecine Sciences, (D. Schwarz), 1977), which is based on a comparison of the averages, and allows the comparison of the values of two independent samples x and y.

[0310] This test is implemented as follows: all of the values of the two groups x and y to be compared are classified in increasing fashion. A rank is then allocated to each value, and the sum of the ranks is calculated. Wx and Wy are then obtained. A reference value called (Wy), (theoretical value in the null hypothesis where Wx is not different from Wy) is then calculated and linked by the ratio: n (N+1)/2, with n=number of mice tested in group x and N=number of mice tested in groups x and y.

[0311] If Wx is less than (Wy), (low residual level of vaccinia virus in the mice), then it can be concluded that the neutralization resulting from the vaccination is significantly effective.

[0312] If we take the example of the group AdNS3NS5b denoted x compared with the group AdβGal denoted y, we obtain the following values:

\[ Wx = 1+2+4+6+8+11+13+14 = 59 \] (8 mice tested)
\[ Wy = 3+5+7+9+10+12+14+15+16+17 = 91 \] (8 mice tested)

[0313] Under the null hypothesis, Wx is not different from Wy, the expected value is: \[ (Wy) = \frac{(N+1)^2}{4} = \frac{(8+1)^2}{4} = 68 \] (8 mice tested)

[0314] Wx < (Wy), which signifies that the values obtained in the group AdNS3NS4NS5b are smaller than those obtained in the group AdβGal and that the neutralization resulting from the vaccination is significantly effective.

[0315] The statistical values for the other groups of mice are indicated in Table 1 below:

<table>
<thead>
<tr>
<th>Group/AdpGa1</th>
<th>Wx</th>
<th>(Wy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdNS3NS4 + NS5b</td>
<td>52</td>
<td>68</td>
</tr>
<tr>
<td>AdNS3NS4 + NS5b + NS5a</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>AdNS3NS4 + NS5b + CE1E2</td>
<td>74</td>
<td>68</td>
</tr>
</tbody>
</table>

[0316] The values in Table 1 above show that only a vaccination of the mice by a combination of the Adenoviruses NS3NS4 and adenovirus NS5b is capable of inducing a significant neutralization of the replication of the vaccinia virus used in the trial with respect to the group of control mice vaccinated by AdβGal. The vaccinations carried out using the combinations comprising (AdNS3NS4+AdNS5b+AdNS5a) or (AdNS3NS4+AdNS5b+AdCE1E2), do not result in a significant difference compared with the group of control mice immunized by AdβGal.

[0317] These results therefore make it possible to demonstrate, unexpectedly, the superior protection of a vaccination combining the polyprotein NS3NS4 and the polypeptide NS5b.

[0318] 5. Confirmation of the Protection of a Vaccination Combining the Polyprotein NS3NS4 and the Polypeptide NS5b Expressed Jointly by the Same Vector

[0319] The recombinant vaccinia virus titre was determined for 3 groups of 8 mice immunized by the following combinations of adenoviruses: AdNS3NS4AdNS5b (1st group), AdNS3NS4+AdNS5b (2nd group) and AdβGal (3rd group).
The results, given in FIG. 7, are treated statistically on the basis of the Wilcoxon Mann-Whitney non-parametric test as described in the previous experiment.

The statistical values for groups 1 and 2 compared to the control group AdpGal are indicated in Table 2 below:

<table>
<thead>
<tr>
<th>Group/AdpGal</th>
<th>Wa</th>
<th>( (Wa)_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdNS3NS4NS5b</td>
<td>49</td>
<td>68</td>
</tr>
<tr>
<td>AdNS3NS4+NS5b</td>
<td>53</td>
<td>68</td>
</tr>
</tbody>
</table>

The values in Table 2 above show that the vaccination of the mice by an adenovirus coding both for the three antigens NS3, NS4 and NS5b, like the combination of the Adenovirus NS3NS4 and Adenovirus NS5b, is capable of inducing a significant neutralization of the replication of the vaccinia virus used in the trial with respect to the group of control mice vaccinated by the AdenoGal. This result confirms the protection of a vaccination combining the polyprotein NS3/NS4 and the polypeptide NS5b expressed jointly by the same vector.

EXAMPLE 4

Accelerated Immunization Schedule Induces Potent, Long-Lasting and Cross-Protective T Cell Response

The MVA vectored vaccine candidate expressing three viral antigens described in Example 2 was evaluated in HLA-class 1 transgenic mouse models for its ability to stimulate CD8+ and CD4+ mediated responses. An accelerated (3 weekly-based) vaccination induced specific CD8+ T cells harboring two effector functions (cytolytic activity—both in vitro and in vivo—and production of IFN-γ) and well as specific CD4+ T cells recognizing all three viral antigens. Responses were long lasting (6 months), boostable by a 4th vaccination and cross-protective as demonstrated in a surrogate Listeria-based challenge assay.

1. Introduction

Approximately 3% of the world’s population is infected with hepatitis C virus (HCV) (Shepard et al., 2005, Lancet 5558-5567) and about 80% of infected people develop a chronic infection leading to liver failure in 4% of cases. Standard treatment combining interferon α (IFNα) and ribavirine is effective in about half of the treated patients, however associated with significant toxicity and cost, and remains counter-indicated in a non-neglectable number of cases. Novel therapies are in development, mainly targeting the viral protease or polymerase (Dev et al., 2004, Current Gastroenterology Reports 677-686). However, preliminary clinical data indicate that these new antivirals display low efficiency when used as stand-alone therapy, and it is becoming clear that the HCV therapeutic field is moving towards a complex association of multiple, costly drugs. The need for alternative therapeutic strategies, relying on complementary mechanisms than those currently exploited by antiviral molecules candidate, is well recognized.

Studies in humans and chimpanzees have indicated that failure to generate broad and long-lasting HCV specific CD4+ and CD8+ T lymphocytes-mediated immune responses during the acute phase of infection correlates with development of chronicity (Shoukry et al., 2004, Annual Review of Microbiology, 58391-58424). Conversely, patients displaying a functional and maintained Th1 CD4+ T lymphocyte-mediated response, associated with the mounting of matured and multifunctional effector CD8+ T lymphocytes, exert a more efficient control of viremia and are prone to evolve towards recovery (Lauer et al., 2004, Gastroenterology 127(3), 924-936; Uribandi et al., 2001, Hepatology 33, 1533-1543; Lechner et al., 2000, J. Exp. Med. 191, 499-512; Thimme et al., 2001, J. Exp. Med. 194, 1395-406; Bowen et al., 2005, Nature 436, 946-52; Cox et al., 2005, Hepatology 42, 104-112). Multiple studies have established that non structural antigens, and in particular NS3, are the preferential targets of responses associated with natural or therapeutic viral clearance (Vertuani et al., 2002, Eur. J. Immunol. 32, 144-54; Diepolder et al., 1997, J. Virol. 71, 6011-9; Smyk-Pearson et al., 2006, J. infect. Dis. 194, 454-63). In contrast, although the field is moving quickly due to novel assays recently developed, the contribution of anti-HCV antibodies in infection outcome remains controversial as these antibodies are typically present in face of ongoing chronicity (Bartosch et al., 2005, Proc. Natl. Acad. Sci. USA, 100, 14199-204; Logvinoff et al., 2004, Proc. Natl. Acad. Sci. USA 101, 10149-54; Maunier et al., 2005, Proc. Natl. Acad. Sci. USA 102, 4560-5).

Over the last ten years, a wide variety of HCV vaccine efforts have been pursued. Various formulations, ranging from classical adjuvanted-recombinant proteins to dendritic cell-based vaccines, have been tested in mice, macaques and for a few, in chimpanzees (Martin et al., 2006, Drug Discovery Today 3206-9). It is striking to observe that only a few vector-based vaccines have been evaluated so far such as recombinant DNA (Forns et al., 1999, Vaccines 17, 1992-2002; Rollier et al., 2004, J. Virol. 78, 187-96), recombinant bacteria (Wademeyer et al., 2001, Gastroenterology 121, 1158-66) or adenoviruses (Arribilaga et al., 2002, Vaccine 21, 202-210; Fopgeri et al., 2006, Nature Medicine 12, 190-7). Most surprisingly, one of the safest known vaccine vector used to date in the clinic, namely the modified non-replicative vaccinia virus Ankara strain (MVA), has seldom been evaluated towards the development of HCV vaccines. This highly attenuated strain of vaccinia virus, that has been used in the campaign for eradication of smallpox, has demonstrated a safety profile in more than 100,000 people (Mayr et al., 1978, Zentrab. Bakteriol. 167, 375-90; Mahnel et al., 1994, Bert. Muench. Tieraerztl. Wochenschr. 107, 253-6). In the case of HCV, only two pre-clinical studies based on MVA vaccines have so far been reported: one describing MVA candidates expressing HCV envelope glycoproteins E1 and E2, either as wild-type or membrane targeted immunogens (Abraham et al., 2004, Vaccine 22, 3917-28), the other reporting on a vaccine combining two MVA expressing the three structural proteins (Core, E1 and E2) as well as the non structural protein 3 (Rollier et al., 2004, J. Virol. 78, 187-96). However, encouraging results with MVA-based vaccines have been observed for example, in the field of HIV or malaria vaccine development, where numerous studies involve MVA vaccine candidates either used alone or in prime-boost combinations (Hanke et al., 1998, J. Gen. Virol; 79, 83-90; Gilbert et al., 2002, Vaccine 20, 1039-45; Prieur et al., 2004, Proc. Natl. Acad. Sci. USA 101, 299-5). These studies have run from evaluations performed in HLA-A2 transgenic murin models to small non-
human primates and to clinical trials (Hanke et al., 2007, J. Gen. Virol. 88, 1-12; Webster et al., 2005, Proc. Natl. Acad. Sci. USA 102, 4836-41). Another poxvirus that has been tested in the HCV vaccine field is an HCV recombinant canarypox virus reported to induce potent T cell immune responses although this candidate has been only tested in a DNA prime-canarypox virus boost regimen (Puncholi et al., 2000, J. Infect. Dis. 182, 18-27). The superior safety profile of MVA combined with its powerful immunogenic potential, argue unambiguously in favour of developing a potent HCV MVA-based vaccine, both for prophylactic and therapeutic application.

[0327] With the aim to develop a safe, poly-antigenic, T cell-based HCV vaccine, we have engineered and preclinically evaluated a recombinant MVA vaccine candidate encoding for HCV non structural (NS) proteins NS3, NS4 and NS5B. We report here that an accelerated schedule of vaccination using this vaccine is able to induce CD4+ and CD8+ T lymphocytes-mediated responses targeted at all three vaccine immunogens and recognizing class I T cell epitopes recognized during the natural infection. Potent and specific CD8+ T cell-mediated responses are long lasting (detectable up to 6 months) and can be efficiently recalled when boosted at a later time with the original MVA NS3-NS5B. Using a challenge model based on HCV recombinant Listeria monocytogenes that can infect liver, we show that the accelerated schedule of vaccination with the MVA NS34-NS5B results in in vivo, cross-protective responses.

2. Materials and Methods

2.1. Synthetic Peptides and Recombinant Proteins

[0328] All synthetic peptides and recombinant proteins used were derived from a genotype 1b sequence (HCV-JA) (Kato et al., 1990, Proc. Natl. Acad. Sci. USA 87, 9524-8). Peptides (Eurogentec) were derived from NS3: CVNGVCWTV (referred as CVN, corresponding to aa 1073 to 1081 on the HCV polyprotein; SEQ ID NO: 28), GLLGCITL (GLL, an 1038 to 1047; SEQ ID NO: 24), KLTGGLN (KLT, an 1406 to 1415; SEQ ID NO: 29), WPAPPGARSM (WPA10, an 1111 to 1121; SEQ ID NO: 30), LSRPRVSYYK (LSV10, an 1152 to 1162; SEQ ID NO: 31) or NS5B: ALEYDVSTL (ALEY, an 2594 to 2602; SEQ ID NO: 25) antigens. A peptide derived from the HCV Core: CVNGVCWTV (referred as CVN, corresponding to aa 1073 to 1081 on the HCV polyprotein; SEQ ID NO: 28), GLLGCITL (GLL, an 1038 to 1047; SEQ ID NO: 24), KLTGGLN (KLT, an 1406 to 1415; SEQ ID NO: 29), WPAPPGARSM (WPA10, an 1111 to 1121; SEQ ID NO: 30), LSRPRVSYYK (LSV10, an 1152 to 1162; SEQ ID NO: 31) or NS5B: ALEYDVSTL (ALEY, an 2594 to 2602; SEQ ID NO: 25) antigens. A peptide derived from the HCV Core: DLMGYIPLV (DLM, aa 132 to 140; SEQ ID NO: 27) was used as irrelevant peptide. Peptides were dissolved in 100% DMSO at a concentration of 10 mM and stored at -20°C until use. Recombinant NS3 helicase (aa 1192 to 1457) and NS5B (aa 2420 to 2989) proteins were expressed in-house in E. Coli and produced endotoxin-free with a purity >95%. Recombinant NS4 protein was obtained from Mikrogen. Tetanus toxoid (TT, Sanofi Pasteur) was used as irrelevant protein.

2.2. Construction of Recombinant MVA NS34-NS5B

[0329] Plasmid used for homologous recombination in the so-called deletion III corresponding site of the MVA genome was based on plasmid pTG1 (Braun et al., 2000, Gene Ther. 7, 1447-57). Flanking sequences (BRG3 and BRD3) surrounding the deletion III were amplified by PCR from MVA NS3 DNA (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. USA 89, 10847-51). The transfer plasmid also contained a fusion between the Aequora victoria enhanced Green Fluorescent protein (eGFP gene, isolated from pEGFP-C1, Clontech) and the E. coli xanthine-guanine phosphoribosyltransferase gene (gpt gene) under the control of the early/late vaccinia virus synthetic promoter p12/8K-7.5 (kindly provided by R. Wittek, University of Lausanne). Synthesis of xanthine-guanine phosphoribosyltransferase enabled GPT recombinant MVA to form plaques in a selective medium containing mycophenolic acid, xanthine, and hypoxanthine. eGFP enables the visualisation of recombinant MVA plaques. When the clonal selection was achieved, the selection marker eGFP-GPT, placed between two homologous sequences in the same orientation, was eliminated by several passages without selection. Gene sequences coding for NS3NS4 and NS5B from HCV genotype 1b HCV-JA strain were amplified by PCR. NS3NS4 gene was inserted in the transfer plasmid downstream the pHR5 promoter (Rosel et al., 1986, J. Virol. 60 436-49) giving rise to pTG16639. NS5B gene was inserted downstream the p7.5K promoter (Cochran et al., 1985, J. Virol. 54, 30-7) in the same orientation as NS3NS4 gene in pTG16639, giving rise to the final transfer plasmid pTG16643. Generation of MVA NS34-NS5B (MVATG16643) was performed by homologous recombination in primary chicken embryo fibroblasts (CEF): pTG16643 was transfected according to the standard calcium phosphate DNA precipitation on to CEF previously infected with MVA N33 at a MOI of 0.1 pfu/cell. Viral selection was performed by three rounds of plaque purification in the presence of a selective medium containing mycophenolic acid, xanthine and hypoxanthine on CEF, then the selection marker was eliminated by passage in non-selective medium. Absence of contamination by parental MVA was verified by PCR.

2.3. In Vitro Expression Studies

[0330] Expression of NS3, NS4 and NS5B antigens was examined by immunofluorescence and flow cytometry following MVA NS34-NS5B infection of human Huh-7 hepatoma cells. For immunofluorescence analysis, glass coverslips were placed into 6-well plates treated with 0.2% Gelatin for 10 min prior to plating Huh-7 cells (10 6 per well) into wells. Cell monolayers were infected with MVA vectors (MVA NS34-NS5B or MVA N33 as negative control) at a MOI of 0.33. Twenty four hours later, coverslips were washed in PBS, fixed with 4% PFA and permeabilized with 0.1% Triton X-100 in PBS. Primary antibodies were applied for 1 h at room temperature (rabbit polyclonal anti-NS4B serum and murine monoclonal anti-NS5B antibody 5B12 which kindly provided by R. Bartenslebinger and D. Moradpour respectively). Alexa-Fluor 488 chicken anti-mouse IgG (Molecular Probes) and Cy3-conjugated anti-rabbit IgG (Molecular Probes) and Cy3-conjugated anti-rabbit IgG sheep (Fab)2 (Sigma) were then added for 30 min. Coverslips were mounted in 80% glycerol in presence of 10 µg/ml Hoechst and strips were observed with Carl Zeiss Axioplan microscope. Images were taken with Axiocam Color digital camera. For flow cytometry analysis, 10 6 Huh-7 cells per well, plated into 6-well plate, were infected with MVA vectors at a MOI of 1. Twenty four hours later, cells were harvested, fixed with Cytofix/Cytoperm reagent (Becton Dickinson) for 10 min and washed with PermWash reagent (Becton Dickinson). Staining was performed using monoclonal anti-NS3 antibody 8D5E1 (BioMerieux), anti-NS4B antibody 1B12A3 (BioMerieux) and anti-NS5B antibody 5B12 (provided by D. Moradpour) added to 2.10 5 cells for 30 min at room temperature. PE-conjugated rabbit anti-mouse IgG (Dako) was then added for 30 min at room...
temperature. Cells were resuspended in 1% FCS-PBS and analyzed by flow cytometry using a FacsCalibur cytometer (Becton Dickinson).

2.4. Mice

[0331] Commercial Balb/c (Charles River) and H-2 class I knockout HLA-A2.1 and HLA-B7.2 transgenic mice were used. HLA-A2.1 mice expressed a transgenic monoclonal histocompatibility class I molecule in which the C-terminus of the human β2m is covalently linked to the N-terminus of a chimeric heavy chain (HLA-A2.1 α1+α2, H-2Dα α3 transmembrane and intracytoplasmic domains) (Pascolo et al., 1997, J. Exp. Med. 185, 2043-51). HLA-B7.2 mice expressed a chimeric HLA-B*0702 heavy chain composed of human HLA-B*0702 α1-2 domains with a murine H-2 Db (3 domain (Rohrlich et al., 2003, International Immunol. 15, 765-72). Mice were hosted at the PBES (Plateau de Biologie Experimentale de la Souris, Lyon) in appropriate animal care facilities and handled in accordance with international guidelines required for experiments with animals.

2.5. Immunization Protocols

[0332] Six to 8 weeks-old mice (2 to 6 per group) were used in each experiment. First, two MVA immunization schedules were compared. Mice received subcutaneous (sc) injections at the tail base with 107 pfu of MVA NS34-NS5B or MVA N33, either 3 injections at 3 weeks-interval (schedule 1) or 4 injections, 3 at 1 week-interval and the 4th one 3 weeks later (schedule 2). A schedule including 3 injections at 1 week-interval was then selected for additional experiments. For analysis of recall memory responses, mice received a 4th MVA injection at 6 months (week 27) after the first immunization.

2.6. ELISPOT Assays

[0333] Splenocytes (2×10^6 cells per well), treated with red blood cell lysis buffer (Sigma), were cultured in triplicate wells for 40 h in multiscreen nitrocellulose-bottomed plates (Millipore) coated with anti-mouse IFNγ monoclonal antibody (Pharmlink) in complete αMEM culture medium (GIBCO BRL) supplemented with murine recombinant IL-2 at 10 U/ml (Pedro-Tech EC LTD) alone as negative control or with 10 µM of peptide or 2 µg/ml of protein. After overnight incubation, GolgiStop (Becton Dickinson) was added and prepared to final concentration for 6 h. Cells were then harvested in V-bottom 96-well plates and washed with 1% FCS-PBS. Staining was realized using monoclonal antibodies (MAB) against CD3 (hamster MAB anti-CD3ε-PE) and CD8 (rat MAB anti CD8α-APC) (all from Becton Dickinson) in 1% FCS-PBS for 15 min at room temperature. After washing, cells were fixed and permeabilized with Cytofix/Cytoperm (Becton Dickinson) and washed twice with Perm/Wash solution. Anti-mouse IFNγ-Alexa488 antibodies (Becton Dickinson) were added for 15 min at room temperature and after washing, cells were resuspended in PBS and analyzed by flow cytometry. CD3ε+, CD8α+ cells were gated and represented on IFNγ-Scatter-dot-plot and percentages of IFNγ+CD8+ T cell population were determined.

2.8. In Vitro and In Vivo Cytotoxic T Cell (CTL) Assays

[0335] Conditions for in vitro CTL assays have been described previously (Brinster et al., 2001, Hepatology 34, 1206-17). Briefly, after restimulation on day 5, CTL assays were performed on day 7 using stimulated cells as effectors. As target cells, 51Cr-stained EL4S3-Rob HHD cells were used either loaded with 10 µM of the selected peptide or unloaded (negative control). Spontaneous and total lysis were determined from wells containing target cells, loaded with peptide or not, either in medium alone or in lysis buffer (1N HCl), respectively. Specific cytotoxicity was calculated using the formula: (release in assay—spontaneous release)/total lysis—spontaneous release)×100. For each effector/target ratio, data are expressed as the mean of duplicate results. A response was considered positive if the percentage of specific lysis was greater than 20% and at least 10% superior to that obtained for MVA N33 immunized mice.

[0336] In vivo CTL assays were performed as described (Beloeil et al., 2003, J. Immunol. 171, 2995-02), with minor modifications. Briefly, splenocytes suspensions were obtained from syngeneic mice and adjusted to 20x10^6 cells/ml after lysis of red blood cells. One half of the cells were incubated with GLL peptide at 10 µM final concentration for 1 h at 37° C, whereas the second fraction was left unpulsed. 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) was then added at 1 µM (CFSElow) to unpulsed cells and at 10 µM (CFSEhigh) to peptide pulsed cells for 10 min. After washing, both populations were mixed and 20x10^6 total cells were transferred to mice by retro-orbital intravenous injection. Thus, CFSEhigh population represented specific targets supposed to be lysed by CTL and CFSElow population was an internal reference allowing assay normalization. Splenocytes from recipient mice were analyzed 24 h later by flow cytometry to detect the CFSE-labeled target cells. The ratio between peptide-pulsed targets and unpulsed targets injected in a given mouse (ratio R=number of CFSEhigh cells/number of CFSElow cells) was calculated. The percentage of specific lysis, that normalized cytolytic activity between MVA NS34-NS5B and MVA N33 immunized mice (control), was determined by the following formula: % of specific lysis=[1—(R immunized/R control)]×100% where R control is the highest ratio R obtained for 2 MVA N33 injected mice.
2.9. Protective Immunity in Surrogate Challenge Model

[0337] Protection was assessed using a surrogate challenge model based on a recombinant Listeria monocytogenes strain producing an HCV NS3 protein derived from a genotype 1a sequence (HCV-1 isolate): TC-LNS3 (Simon et al., 2003, Infection and Immunity 71, 6372-80). MVA NS34-N535B immunized mice received an i.v. injection with 1 LD₅₀ (9.10⁷ colony-forming units (CFU) for mice with a C57BL/6 background like HLA-A2 mice, 3.10⁷ CFU for Balb/c mice) of TC-LNS3 in 100 μl of PBS 2 weeks following the last MVA immunization. As negative control group, MVA N33 immunized mice were used and as positive control group, we used mice that were immunized 1 or 2 times at 2 weeks-interval with 0.05 to 0.1 LD₅₀ of TC-LNS3 (immunization dose). Two days after bacterial challenge, spleens and livers were removed from individual mice, weighted, homogenized and serially diluted in PBS/0.1% Triton. These dilutions were plated out on brain heart infusion agar. Following 2 to 3 days at room temperature, the number of CFU was calculated and results given in Log CFU/mg of tissue values from individual mouse.

2.10. Statistical Analyses

[0338] Analyses of CTL responses, IFNγ ELISPOT responses and protective effect were conducted by using a Mann-Whitney test.

3. Results

3.1. Design and In Vitro Expression of a Single Recombinant MVA Encoding for HCV NS3, NS4 and NS5B Proteins

[0339] Two recombinant MVA vectors, encoding each HCV NS3NS4 proteins under the ph5r promoter and NS5B protein under the p7.5 promoter, were designed. Both expression cassettes were cloned in the deletion III of the MVA backbone, either in same or in opposite orientation. Western blot analysis revealed an enhanced expression of all three cloned antigens when the two expression cassettes were inserted in the same orientation (data not shown). Thus, the MVA vector containing both expression cassettes in the same orientation, referred to as MVA NS34-N535B, was selected for further studies. In vitro NS3, NS4 and NS5B antigen expression was characterized by flow cytometry and immunofluorescence analyses following MVA NS34-N535B infection of Huh-7 cells. Flow cytometry analyses showed clear expression of the three antigens (FIG. 8). While the percentage of expressing cells following infection appeared identical for the three encoded antigens, measure of mean fluorescence intensity (MFI) suggested a weaker expression of the NS5B likely due to a lesser strength of the p7.5 promoter compared with the ph5r promoter. Co-localisation percentage of expressing cells following infection appeared similar for the three encoded antigens when the two expression cassettes were cloned in the deletion III of the MVA backbone, either in same or in opposite orientation. A weak but specific cytopathic effect (CPE) was observed against Huh-7 cells infected with MVA NS34-N535B (FIG. 9A), while lower percentages of CPE were observed against Huh-7 cells infected with MVA N33 (wild type virus used as negative control). In vitro infections were repeated at least three times at 2 weeks-interval following 3 infections at 1 week-interval, either 2 weeks after the 2nd and the 3rd injection for schedule 1 (week 6 and 9) or 2 weeks after the 3rd and 4th injection for schedule 2 (week 5 and 8) (FIG. 9). As shown in FIG. 9A, both injection schedules resulted in the induction of IFNγ-producing cells specific for two HLA-A2 restricted epitopes (GLL and KLT) located in the NS3 protein and known to be recognized during natural infection (Martin et al., 2004, J. Med. Virol. 74, 397-405; Ward et al., 2002, Clin. Exp. Immunol. 128, 195-205). A similar number of spots was observed with both schedules: an elevated number was observed for the GLL epitope (up to 1000 spots), in agreement with observations describing GLL as a dominant epitope in HLA-A2 mice (Martin et al., 2004, J. Med. Virol. 74, 397-405), while responses were weaker against the KLT epitope (up to 200 spots). Although the frequency of GLL specific positive cells waned with time and was found lower two weeks after the final injection for both schedules (week 9 for schedule 1 and 8 for schedule 2, FIG. 9B), the overall observed number of spots remained comparable for both schedules. The KLT specific response was at that time lost (data not shown). High cytotoxic activity was detected against the GLL peptide using both schedules at the first time point studied (week 6 for schedule 1 and week 5 for schedule 2) (FIG. 10A), while lower percentages of specific lysis were observed after boost (week 9 and 8) (FIG. 10B). A weak but specific cytotoxic activity targeted at the subdominant NS5B ALY epitope was detected for 2 mice after 3 injections (FIG. 10A) and 1 mouse after 4 injections following schedule 2 (FIG. 10B). Overall, these results suggest that 2 injections at 3-weeks intervals induced similar NS3 specific cytotoxic activities and frequencies of IFNγ-producing T cells than those obtained by 3 injections at 1 week-interval. An additional booster injection, independent of the schedule (the 3rd injection for schedule 1 or the 4th for schedule 2), did not enhance these responses. Further experiments were performed based on vaccination schedule 2, referred to as “accelerated” vaccination schedule.

3.2. An Accelerated Vaccination Schedule with the MVA NS34-N535B Induces Specific CD8+ T Cells Displaying Two Effector Functions Comparable with those Obtained Following a More Lengthy Classical Schedule

[0340] The mounting of immune responses following MVA-based vaccination has typically been analyzed following 2-3 injections of candidate vaccines performed 2-4 weeks apart (Gilbert et al., 2002, Vaccine 20, 1039-45; Vazquez-Blomquist et al., 2004, Biotechnol. Applied Biochem. 39, 313-8). Yet, it may be worthwhile to indeed quickly mount specific immune responses to try to minimize impact of vector-developed anti-immunity as well as to have a faster impact on viral load and/or disease evolution in cases of therapeutic vaccination. Based on recent, highly encouraging data obtained in the clinics with a HPV (human papilloma virus)-based MVA vaccine administered according to an accelerated schedule (Transgene Abstract, EUROGIN 2006), we decided to first compare side by side two different schedules of vaccination. HLA-A2 transgenic mice received, subcutaneously, either 3 injections at 3-weeks-interval (schedule 1) or 3 injections at 1-week-interval followed by a 4th injection, 3 weeks later (schedule 2) of 10⁷ pfu MVA NS34-N535B or MVA N33 (wild type virus used as negative control). Induced CD8+ T cell responses were investigated by in vitro CTL and IFNγ ELISPOT assays at two time points for each schedule, either 2 weeks after the 2nd and the 3rd injection for schedule 1 (week 6 and 9) or 2 weeks after the 3rd and 4th injection for schedule 2 (week 5 and 8) (FIG. 9). As shown in FIG. 9A, both injection schedules resulted in the induction of IFNγ-producing cells specific for two HLA-A2 restricted epitopes (GLL and KLT) located in the NS3 protein and known to be recognized during natural infection (Martin et al., 2004, J. Med. Virol. 74, 397-405; Ward et al., 2002, Clin. Exp. Immunol. 128, 195-205). A similar number of spots was observed with both schedules: an elevated number was observed for the GLL epitope (up to 1000 spots), in agreement with observations describing GLL as a dominant epitope in HLA-A2 mice (Martin et al., 2004, J. Med. Virol. 74, 397-405), while responses were weaker against the KLT epitope (up to 200 spots). Although the frequency of GLL specific positive cells waned with time and was found lower two weeks after the final injection for both schedules (week 9 for schedule 1 and 8 for schedule 2, FIG. 9B), the overall observed number of spots remained comparable for both schedules. The KLT specific response was at that time lost (data not shown). High cytotoxic activity was detected against the GLL peptide using both schedules at the first time point studied (week 6 for schedule 1 and week 5 for schedule 2) (FIG. 10A), while lower percentages of specific lysis were observed after boost (week 9 and 8) (FIG. 10B). A weak but specific cytotoxic activity targeted at the subdominant NS5B ALY epitope was detected for 2 mice after 3 injections (FIG. 10A) and 1 mouse after 4 injections following schedule 2 (FIG. 10B). Overall, these results suggest that 2 injections at 3-weeks intervals induced similar NS3 specific cytotoxic activities and frequencies of IFNγ-producing T cells than those obtained by 3 injections at 1 week-interval. An additional booster injection, independent of the schedule (the 3rd injection for schedule 1 or the 4th for schedule 2), did not enhance these responses. Further experiments were performed based on vaccination schedule 2, referred to as “accelerated” immunization schedule.

3.3. Accelerated Vaccination with the MVA NS34-N535B Induces Significant Percentage of CD8+ T Cells Producing IFNγ and Displaying Potent In Vivo Lytic Activity

[0341] To further characterize the immunogenicity of the accelerated vaccination schedule, we investigated, in HLA-A2 transgenic mice, the capacity of induced CD8+ T cells to produce IFNγ by intracellular IFNγ cytokine staining (ICS) as well as their cytotoxic potential in vivo. ICS analysis, performed 2 weeks after the 3rd injection (FIG. 11A), indi-
injected that all 4 immunized animals displayed a high percentage of GLL specific CD8+ T cells producing IFNγ (ranging from 1.13 to 2.3%, median percentage of 1.7%). We examined the in vivo killing capacity of specific effector CD8+ T cells by transferring CFSE-labeled target splenocytes pulsed with GLL peptide into MVA NS34-NS5B or MVA NS3 immunized mice, 2 weeks after the 3rd injection. After transfer, GLL-pulsed targets were eliminated efficiently in the MVA NS34-NS5B immunized mice (FIG. 11B: lysis of 15%, 45%, 65% and 78% for each mouse, respectively) in a 20 h assay, whereas CTL activity was undetectable in control mice (not shown). Overall, these results confirm that CD8+ T cells induced in mice immunized with the MVA NS34-NS5B on a weekly basis have acquired significant capacity to produce IFNγ and to display a cytolytic activity in vivo.

3.4. Accelerated Vaccination with MVA NS34-NS5B is Capable to Induce Responses Targeting HLA-B7 Restricted Epitopes

It was recently reported that HLA-B restricted immune responses may play a major role in the outcome of HCV infection (Neumann-Ilaelaein et al., 2006, Hepatology 43, 563-72), similar to what has been described for HIV and EBV (Frahm et al., 2005, J. Virol. 79, 10218-25; Kiepiela et al., 2004, Nature 432, 769-75; Bihl et al., 2006, J. Immunol. 176, 4094-101). To address the capacity of the MVA NS34-NS5B to prime, in vivo, cellular immune responses restricted by an HLA-B molecule, HLA-B7 transgenic mice were immunized following the accelerated schedule and CD8+ T cell responses investigated using IFNγ ELISPOT assay. As shown in FIG. 12, the vaccine was able to prime significant IFNγ-producing T cells specific of two HLA-B7 restricted epitopes that are the only NS3 HLA-B7 epitopes so far described in HCV infection, namely WPA10 and LSP10 (Martin et al., 2004, J. Med. Virol. 74, 397-405). In 4 (WPA10) or 5 (LSP10) out of 5 immunized animals, IFNγ-producing T cells were detected with a high frequency for WPA10 (median value: 305 spots/10^6 splenocytes), and with a weaker frequency for LSP10 (median value: 160 spots/10^6 splenocytes). These results demonstrate the capacity of the MVA NS34-NS5B to induce, in addition to HLA-A2 restricted responses, HLA-B7 restricted responses specific of epitopes recognized in a HCV natural infection.

3.5. CD4+ T Cell Responses Specific of all Three Immunosgens Expressed by the MVA NS34-NS5B are Induced Following Accelerated Immunization

Due to the critical role of CD4+ T cell mediated responses in determining the outcome of HCV infection, it is of obvious importance that HCV vaccine candidates harbour the capacity to generate such responses. We assessed the ability of the MVA NS34-NS5B to induce, following the “accelerated” schedule of administration, specific CD4+ T cell responses in Balb/c mice as such responses were difficult to detect in HLA-A2 or -B7 mice, both displaying a C57Bl6 genetic background (data not shown). The CD4+ T cell responses were evaluated 2 weeks after the 3rd immunization by IFNγ-ELISPOT and ICS analysis performed on total splenocytes, the CD4+ T cell positive fraction as well as the effluent fraction obtained after positive selection. As shown in FIG. 13, IFNγ-producing cells specific of each NS3, NS4 and NS5B protein were detected using the purified CD4+ T cell fractions obtained from immunized mice, while no signals could be seen in control animals. A weak IFNγ ELISPOT response specific of NS4 was also detected using the total splenocyte fraction. ICS analysis led to similar results (data not shown). Overall, these data reveal that a weekly administration of the MVA NS34-NS5B is able to induce CD4+ T cells specific of all three expressed antigens.

3.6. CD8+ T Cell Specific Responses Induced Following Accelerated Vaccination with MVA NS34-NS5B are Long Lasting and can be Boosted

A key feature common to potent vaccines is their potential to induce long lasting, memory responses. To evaluate the longevity of MVA NS34-NS5B CD8+ T cell responses induced following accelerated vaccination, a two-steps experiment was performed. The first step evaluated the longevity of responses by performing CTL and IFNγ ELISPOT assays at 1 month (week 5), 2 months (week 10) and 6 months (week 27) post primary injection. In a second step, the capacity of a 4th MVA NS34-NS5B injection to recall memory responses was explored, using the same assays, 2 weeks after a recall injection performed 6 months post primary vaccination (week 29). CTL responses induced at week 5, 10, 27 and 29 are represented in FIG. 14. Strong CTL responses specific of the GLL peptide were detected in all mice, at each time point studied, even at low effector/ target cell ratios (median values at an E/T ratio of 11/1: 68% of lysis at week 5, 73% at week 10, 47% at week 27). Responses detected 2 weeks after the 4th MVA NS34-NS5B injection remained similar to those measured before this injection (median value at an E/T ratio of 11/1: 51% of lysis).

3.7. Accelerated Vaccination with the MVA NS34-NS5B Induces In Vivo Protective Responses in a Surrogate Challenge Assay

Because hepatocytes represent the main replication site for HCV, one of the goals of a HCV vaccine is to induce T cells able to migrate to the liver and destroy antigen expressing cells. We used a surrogate challenge model...
mimicking to some extent HCV infection to investigate the capacity of the MVA NS34-NS5B vaccine at generating such desirable response. The challenge agent, a recombinant Listeria monocytogenes expressing the NS3 protein from an HCV genotype 1a strain (referred to as TC-LNS3), (Simon et al., 2003, Infection and Immunity 71, 6372-80), was used as these bacteria are able to infect and replicate within hepatocytes (Jiang et al., 1997, 158, 287-93). Numerous studies have shown that a strong antigen-specific CD8+ T cell response is required for protection against L. monocytogenes infection (Simon et al., 2003, Infection and Immunity 71, 6372-80; Baldrige et al., 1990, Infection and Immunity 58, 654-58). HLA-A2 transgenic mice were immunized according to the accelerated immunization schedule with MVA NS34-NS5B or MVA N33. A group of mice, immunized intravenously 1 or 2 times at 2-weeks interval with a low immunization dose (0.05 to 0.1 LDso) of TC-LNS3 (able to protect mice against a further infection with a high challenge dose of TC-LNS3 (Simon et al., 2003, Infection and Immunity 71, 6372-80)) was included as positive control-immunized mice. MVA vaccinated mice were challenged intravenously with a high challenge dose of TC-LNS3 (1 LDso) 2 weeks after the 3rd MVA injection. For the positive control group, the challenge was performed 1 week after the 2nd injection of the low TC-LNS3 dose as reported (Simon et al., 2003, Infection and Immunity 71, 6372-80). Two days after challenge, the number of viable bacteria in the spleen and liver of each mouse was determined. Experiments were performed in two strains of mice, HLA-A2 and Balb/c mice. The results presented in FIG. 16A show that MVA NS34-NS5B immunized mice exhibited significantly lower bacterial loads in the spleen (median value: 3.83 Log CFU/mg, p=0.02) following challenge than did MVA N33 immunized mice (5.17 Log CFU/mg). These data are representative of 4 independent experiments. In the experiment shown, bacterial counts were also significantly reduced in the liver of MVA NS34-NS5B injected mice (4.53 Log CFU/ml, p=0.02) compared with those of MVA N33 immunized animals (5.62 Log CFU/ml), although reduction in this organ was lower and not always statistically significant i.e. experiment dependent (data not shown). MVA NS34-NS5B Balb/c-immunized mice demonstrated a significant reduction of bacterial loads in the spleen as compared with MVA N33-immunized mice (median value: 2.28 versus 2.89 Log CFU/mg, p=0.006). In this representative experiment, as typically observed in this strain of mice, reduced bacterial counts were also seen in the liver, however differences were not statistically significant (p=0.05). These original data demonstrate that a weekly administration of the MVA NS34-NS5B can prime immune responses capable of conferring immune protection against subsequent challenge with recombinant L. monocytogenes expressing HCV NS3 protein in two different mouse species. As the mechanism of protection in this model has been shown to involve effector CD8+ T cells (Baldrige et al., 1990, Infection and Immunity 58, 654-58), these data lead us to conclude that the MVA vaccine is capable of inducing such cells, in particular cells displaying the capacity to migrate and exert their function in the livers of animals. The challenge bacteria used contains a genotype 1a N33 while the MVA vaccine is expressing a genotype 1b protein thus demonstrating that cross-protective responses can be generated by the vaccine.

4. Discussion

In the present study, we designed a HCV vaccine candidate based on the vaccine strain MVA expressing three HCV antigens, NS3, NS4 and NS5B, from a genotype 1b viral strain (MVA NS34-NS5B). Injected according to an “accelerated” immunization schedule based on 3 injections performed 1 week apart in various HLA-transgenic or commercial mouse models, we show that this candidate vaccine induces simultaneously specific CD8+ T cells able to produce IFNγ and to lyse cells as well as specific CD4+ T cells. We show that the CD8+ T cell responses induced are long lasting responses, detectable up to 6 months post primary vaccination, and boostable with a 4th immunization. Finally, cross-protective effects of the MVA NS34-NS5B induced responses were demonstrated in two mouse species using a surrogate challenge assay based on a recombinant HCV NS3 Listeria monocytogenes.

Our strategy for the design and development of a HCV vaccine was based on the fundamental observation that broad, effective and sustained T cell-based immunity is associated with a favorable outcome of infection either spontaneously or therapy-induced (Bowen and Walker, 2005, Nature 436, 946-52). Three key elements guided our approach: the choice of vaccine immunogens containing multiple CD4+ and CD8+ T cell-restricted epitopes, the selection of a safe and efficient vector platform and that of a vaccination schedule capable to rapidly mount an efficient T cell immunity.

The three immunogens encoded by our vaccine were selected on different criteria. NS3 emerged as a mandatory antigen as its contribution to the total magnitude of the HCV specific CD4+ and CD8+ T cell responses found in resolved infection appear to be an essential one (Diepolder et al., 1997, J. Virol. 71, 6011-9; Smyk-Pearson et al., 2006, J. Infect. Dis. 194, 545-63). At least one vaccine study has reported the critical role played by NS3 specific Th1 responses in vaccine-mediated control of HCV viremia in the chimpanzee model (Roller et al., 2004, J. Virol. 78, 187-96). In contrast to the great majority of NS3-based vaccine studies, that have included NS3 as a stand-alone immunogen (Arribillaga, 2002, Vaccine 21, 202-10; Wedemeyer et al., 2001, Gastroenterology 121, 1158-66; Jiao et al., 2003, Hepatology 37, 452-60; Wuest et al., 2004, Vaccine 22, 2717-21), we have in this report successfully designed a single, stable recombinant MVA (stability observed up to 6 passages), expressing NS3 in association with two other non structural proteins, NS4 and NS5B. NS3 was co-expressed with NS4 as it has been documented that such co-expression influenced positively the immunogenicity of NS3 (Himoudi et al., 2002, J. Virol. 76, 12735-46). In our configuration, NS5B was expressed as a wild-type antigen and in absence of NS5A. This choice was based on our previous observations indicating that NS5B but not NS5A contained highly immunogenic HLA-A2-restricted T cell epitopes (Himoudi et al., 2002, J. Virol. 76, 12735-46).

MVA was selected over other clinically used vectors for different reasons. No integration of the viral genome in the host DNA is possible as the vacinia virus lifecycle...
takes place entirely in the cytoplasm of cells (Moss, 2001, pp 2849-83 in Fields Virology 4th ed. Lippincott-Raven Press). MVA has been attenuated by more than 570 passages in chicken embryo fibroblasts resulting in the loss of about 15% of its genome (Meyer et al., 1991 J. Gen. Virol. 72, 1031-38). Consequently, MVA is unable to produce mature virions in most mammalian cells due to a block at the stage of virion formation (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. USA 89, 10847-51) that results in a reduced risk of dissemination (Carroll and Moss, 1997, Virol. 244, 365-96) and an increased immunogenicity due to the loss of several anti-immune defense genes (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. USA 89, 10847-51). Controversial data have been reported concerning the influence of preexisting anti-vaccinia virus immunity on MVA-based vaccine efficacy (Wedemeyer et al., 2001, Gastroenterology 121, 1158-66; Ramirez et al., 2000, J. Virol. 74, 923-33; Belyakov et al., 1999, Proc. Natl. Acad. Sci. USA 96, 4512-7). However, if preexisting immunity has indeed a detrimental effect on MVA vaccine efficacy, this appears minimal compared with that observed in the case of adenoviral vectors. Pre-existing anti-adenovirus immunity to the widely clinically used Ad5 vector is indeed well known to considerably affect efficacy of Ad5-based vaccines (Casimoro et al., 2003, J. Virol. 77, 6305-13).

[0350] We have evaluated here an original “accelerated” schedule of vaccination based on different arguments. First, recent very encouraging clinical data have been obtained with a therapeutic HPV-based MVA vaccine administered on a weekly basis (3 times). This phase II trial reports the clearance of HPV viral RNA in women with grade 2/3 dysplasies up to 12 months following vaccination with a MVA expressing the viral E6 and E7 proteins (Transgene Abstr. EUROGIN 2006). These novel data suggest that it may be important, in the optic of development of a therapeutic vaccine, to quickly mount an effective response rather than mounting it over time. Following weekly injection of the HCV MVA NS34-NS5B, we show in mouse models the unambiguous mounting of potent CD8+ T cell responses, displaying two effector functions that are believed to play a role in control of HCV replication (capacity to produce IFNγ and to lyse target cells). In addition, it was particularly interesting to observe that these responses, at least the capacity to produce IFNγ, could be boosted 6 months after the last weekly injection confirming that a long lasting immunological memory state can be developed by a schedule including 3 close MVA immunizations. This feature is a key element both for the development of a preventive and a therapeutic vaccine. It suggests that anti-MVA immunity mounted after the “accelerated” vaccination either waned with time or was not mounted yet to an extent sufficient for affecting vaccine immunity. The capacity of our vaccine to induce long lasting CD8+ responses together with specific CD4+ T cell responses and the fact that overall induced responses were capable to control splenic or hepatic expression of a HCV antigen, are extremely encouraging. Indeed, a recent study performed in a chimpanzee model, has shown that an Ad5 T cell-based vaccine expressing NS3-NS4A-NS5B antigens was capable to elicit non-sterile yet protective immunity in 4 out of 5 challenged animals (Folgori et al., 2006 Nature Medicine 12, 190-7). Protection in this study was correlated with the mounting of T cell responses, in particular with the intrahepatic presence of CD8+ T cells specific of NS3 and NS5 antigens. Interestingly, the immunization scheme in that study was atypical in the sense that 3 adenovirus vaccinations were first performed followed by 3 injections of a DNA vaccine, this latter vaccine was apparently added to improve mounting of specific CD4+ T cell responses that are notoriously weak following Ad5 vaccination. Similar to our observations, this Ad5 vaccine was also shown to induce genotype 1-cross protective responses. Both Folgori et al. (Folgori et al., 2006 Nature Medicine 12, 190-7), and our candidate vaccine contain subtype 1b sequences and are obviously capable for inducing responses cross-reacting with subtype 1a determinants as the challenge strains used in our study and in Folgoris’ involve subtype 1a sequences. This feature is also very encouraging although clearly additional evaluations must be performed to more precisely measure the extent of the cross-reactivity induced.

[0351] In conclusion, we have designed and produced a HCV candidate vaccine based on a clinically approved MVA vaccine vector displaying a high and widely recognized safety profile. This vaccine, which is capable to mount potent, long lasting and cross-protective T cell mediated immune responses is currently entering phase I clinical trial.

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tcc gcc gac ctt ggg gat ctt gtc gca gca gca gca cag ctc gcc 2640
  Pro Ser Ala Asp Leu Val Val Ala Cys Ala Ala Ala Ala Leu Arg Arg His
  865  870  875  880

tgg ggg gcc gac ctc gtc ggc gct gct ggg gct ggg ggg cct ggg ggg ggg Val Gly Pro Gly Ala Val Gly Gly Glu Val Gly Gly Glu Leu Leu
  2688
  885  890  895

tgg gcc ggc ggg ggc ggt gct gtc ctc ggc ggg ggg ggg ggg ggg ggg Val Val Gly Gly Glu Gly Gly Gly Phe Ala Pro Gly Ala Pro Thr His Tyr Leu Pro Glu 2736
  895  900  905  910

gac gcc gcc gca cgt gta act cag atc ctc gcc cag ctc aca ctc acc aca Ser Asp Ala Ala Asp Leu Thr Val Gly Ile Leu Ser Ser Leu Thr Ile 2784
  915  920  925
act cag ctc cag aag ctt cac cag tgg att aat gag gac tgc tcc 
Thr Gln Leu Leu Lys Arg Leu His Gln Trp Ile Asn Glu Asp Cys Ser 
930 935 940
acg cca tgc taa 
Thr Pro Cys 
945
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: sequence coding for NS3NS4
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Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Asp Gly 
20 25 30
Glu Val Glu Val Leu Ser Thr Ala Thr Gln Ser Phe Leu Ala Thr Cys 
35 40 45
Val Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Ser Lys Thr 
50 55 60
Leu Ala Gly Pro Lys Gly Pro Ile Thr Gln Met Tyr Thr Asn Val Asp 
65 70 75 80
Gln Asp Leu Val Gly Trp Pro Ala Pro Gly Ala Arg Ser Met Thr 
85 90 95
Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr Arg His Ala 
100 105 110
Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Gly Ser Leu Leu 
115 120 125
Ser Pro Arg Pro Val Ser Tyr Leu Lys Gly Ser Ser Gly Gly Pro Leu 
130 135 140
Leu Cys Pro Ser Gly His Val Val Gly Pro Ile Leu Arg Ala Ala Val Cys 
145 150 155 160
Thr Arg Gly Val Ala Lys Ala Val Asp Pro Ile Pro Val Glu Ser Met 
165 170 175
Glu Thr Thr Met Arg Ser Pro Val Phe Thr Asp Asn Ser Ser Pro Pro 
180 185 190
Ala Val Pro Gln Thr Phe Glu Val Ala His Leu His Ala Pro Thr Gly 
195 200 205
Ser Gly Lys Ser Thr Lys Val Pro Ala Ala Tyr Ala Ala Glu Gly Tyr 
210 215 220
Lys Val Leu Val Leu Asn Pro Ser Val Ala Ala Ala Thr Leu Gly Phe Gly 
225 230 235 240
Ala Tyr Met Ser Lys Ala His Gly Ile Glu Pro Asn Ile Arg Thr Gly 
245 250 255
Val Arg Thr Ile Thr Thr Gly Gly Pro Ile Thr Tyr Ser Thr Tyr Gly 
260 265 270
Lys Phe Leu Ala Asp Gly Cys Ser Gly Gly Ala Tyr Asp Ile Ile 
275 280 285
Ile Cys Asp Glu Cys His Ser Thr Asp Trp Thr Ile Leu Gly Ile 
290 295 300
Gly Thr Val Leu Asp Gln Ala Glu Thr Ala Gly Ala Arg Leu Val Val
Leu Ala Thr Ala Thr Pro Pro Gly Ser Ile Thr Val Pro His Pro Asn
Ile Glu Glu Val Ala Leu Ser Asn Thr Gly Glu Ile Pro Phe Tyr Gly
Lys Ala Ile Pro Ile Glu Ala Ile Lys Gly Gly Arg His Leu Ile Phe
Cys His Ser Lys Lys Cys Asp Glu Leu Ala Ala Lys Leu Thr Gly
Leu Gly Leu Asn Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val Ser Val
Ile Pro Thr Ser Gly Asp Val Val Val Ala Thr Asp Ala Leu Met
Thr Gly Phe Thr Gly Asp Phe Asp Ser Val Ile Asp Cys Ala Thr Cys
Val Thr Gln Thr Val Asp Phe Ser Leu Asp Pro Thr Phe Thr Ile Glu
Thr Thr Val Val Pro Gln Asp Ala Val Ser Arg Ser Glu Arg Arg Gly
Arg Thr Gly Arg Gly Arg Ser Gly Ile Tyr Arg Phe Val Thr Pro Gly
Glu Arg Pro Ser Gly Met Phe Ser Ser Val Leu Cys Glu Cys Tyr
Asp Ala Gly Cys Ala Trp Tyr Glu Leu Thr Pro Ala Glu Thr Thr Val
Arg Leu Arg Ala Tyr Leu Asn Thr Pro Gly Leu Pro Val Cys Glu Asp
His Leu Glu Phe Trp Glu Ser Val Phe Thr Gly Leu Thr His Ile Asp
 Ala His Phe Leu Ser Gln Thr Lys Glu Ala Gly Asp Asn Phe Pro Tyr
Leu Val Ala Tyr Gln Ala Thr Val Cys Ala Arg Ala Gln Ala Pro Pro
 Pro Ser Trp Asp Gln Met Trp Lys Cys Leu Ile Arg Leu Lys Pro Thr
Leu His Gly Pro Thr Pro Leu Tyr Arg Leu Gly Ala Val Glu Asn
Glu Ile Thr Leu Thr His Pro Ile Thr Lys Phe Val Met Ala Cys Met
Ser Ala Asp Leu Glu Val Val Thr Ser Thr Trp Val Leu Val Gly Gly
Val Leu Ala Ala Ala Ala Tyr Cys Leu Thr Thr Gly Ser Val Val
Ile Val Gly Arg Ile Ile Leu Ser Gly Arg Pro Ala Val Val Pro Asp
Arg Glu Val Leu Tyr Arg Glu Phe Asp Glu Met Glu Gly Cys Ala Ser
His Leu Pro Tyr Ile Glu Gln Met Gln Leu Ala Glu Gln Phe Lys
Gln Glu Ala Leu Gly Leu Leu Glu Thr Ala Thr Lys Gin Ala Glu Ala
Ala Ala Pro Val Val Glu Ser Arg Trp Arg Ala Leu Glu Ala Phe Trp
  725  730  735
Ala Lys His Met Trp Asn Phe Ile Ser Gly Ile Gln Tyr Leu Ala Gly
  740  745  750
Leu Ser Thr Leu Pro Gly Asn Pro Ala Ile Ala Ser Leu Met Ala Phe
  755  760  765
Thr Ala Ser Ile Thr Ser Pro Leu Thr Gln Asn Thr Leu Leu Phe
  770  775  780
Asn Ile Leu Gly Gly Trp Val Ala Glu Leu Ala Pro Pro Ser Ala
  785  790  795  800
Ala Ser Ala Phe Val Gly Ala Gly Ile Ala Gly Ala Ile Gly Ser
  805  810  815
Ile Gly Leu Gly Lys Val Leu Val Asp Ile Leu Ala Gly Tyr Gly Ala
  820  825  830
Gly Val Ala Gly Ala Leu Val Ala Phe Lys Val Met Ser Gly Glu Ala
  835  840  845
Pro Ser Ala Glu Asp Leu Val Asn Leu Leu Pro Ala Ile Leu Ser Pro
  850  855  860
Gly Ala Leu Val Gly Ile Val Cys Ala Ala Ile Leu Arg Arg His
  865  870  875  880
Val Gly Pro Gly Glu Ala Val Gln Trp Met Asn Arg Leu Ile Ala
  885  890  895
Phe Ala Ser Arg Gly Asn Val Ser Pro Thr His Tyr Val Pro Glu
  900  905  910
Ser Asp Ala Ala Arg Val Thr Gln Ile Leu Ser Ser Leu Thr Ile
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Thr Gln Leu Leu Lys Arg Leu His Gln Trp Ile Asn Glu Asp Cys Ser
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Thr Pro Cys
  945
210> SEQ ID NO 3
211> LENGTH: 1779
212> TYPE: DNA
213> ORGANISM: Artificial sequence
220> FEATURE: OTHER INFORMATION: sequence coding for NS5b
220> FEATURE:
221> NAME/KEY: CDS
222> LOCATION: (1)..<(1779)
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gcg gag gag agc aag ttc ccc atc aat cgg tgg atc aac tgg ctg
  96
Ala Glu Glu Ser Lys Leu Pro Ile Asn Pro Leu Ser Asn Ser Leu Leu
  20  25  30
cgt cac cac agt atg gtc tac tcc aca aca ctc cgg agc aag cag cgg act
  144
Arg His His Ser Met Val Tyr Ser Thr Ser Arg Ser Ala Ser Leu
  35  40  45
cgg cag aag aag gtc acc tgt gac aag aag aag gag gtc gcc gac cac
  192
Arg Glu Lys Val Thr Phe Asp Leu Glu Val Val Aep Asp His
  50  55  60
tac cgg gcc gtc ctc aag gag atg aag gog agc cag tcc aca gtt aag
  240
Tyr Arg Asp Val Leu Lys Glu Met Lys Ala Lys Ala Ser Thr Val Lys

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Ser Asn Val Ser Val Ala His Asp Ala Ser Gly Lys Arg Val Tyr Tyr
370 375 380
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Leu Thr Arg Asp Pro Thr Thr Pro Leu Ala Arg Ala Ala Trp Glu
385 390 395 400
400
gtt aga cac act cca gtc acc tcc tgg cta ggc aat atc atc atg tat
Val Arg His Thr Pro Val His Leu Met Thr His Phe Phe Ser
405 410 415
415
gcg ccc acc cta tgg ggc aag att cat atg act cat ttc ttc ttt
Ala Pro Thr Leu Leu Ala Arg Met Ile Leu Met Thr His Phe Phe Ser
420 425 430
430
ttc cta gct cag gag cca ctt gaa aac gcc ctt gat tgg cag atc
Ile Leu Leu Ala Gin Glu Glu Gin Leu Gin Leu Arg Cys Gin Ile
435 440 445
445
tac ggg gcc tgc tac tcc att gaa ctt gac cta cct cag atc
Tyr Gly Ala Cys Tyr Ser Ile Glu Pro Leu Arg Leu Pro Gin Ile Ile
450 455 460
460
gaa cga ctc act gtt ctt agc gca ttt tca ctc gta ggt gac
Gly Arg Leu His Glu Gin Val Ile Tyr Leu Gly Asp
465 470 475 480
480
gtt gat atc aat agg gtt gct tca gtc cag aac atg cag
Gly Glu Ile Asn Arg Val Ala Leu Cys Leu Arg Asn Arg
485 490 495
495
cct cag ctc tgg cag ctc gcc gcg aag gct cgg ggc gca
Pro Leu Arg Val Thr Arg Gin Gin Leu Arg Gin Gin Gin Gin
500 505 510
510
cag cta ggg ggg agg gcc ctc gcc tgg ctc ctt ctt tct gaa gaa
cag ctt gta agg acc aag ctt aac cta ctc a ac ctc cct cgg gat
Gln Leu Gin Gin Gin Arg Gin Gin Gin Gin Gin Gin Gin Gin Gin
515 520 525
525
tgg gcc gta agg acc aag ctt aac cta act cca ctc gct gct gct
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Trp Ala Val Arg Thr Leu Leu Leu Thr Pro Ile Pro Ala Ala Ser
530 535 540
540
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cag cta gac tgg tgc tgg tgg tgc gtt ctt ggt ggt tac aac aag ggg gca
Gln Leu Asp Leu Ser Gly Trp Phe Val Ala Gly Tyr Asn Gly Gly Asp
545 550 555 560
560
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Ile Tyr His Asp Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg
565 570 575
575
tct ctc cta cct gtt ggg gga acc ctt cct aac tgg tgg tgg tgg
Leu Leu Leu Val Gly Gly Val Gly Ile Tyr Leu Leu Pro Asn Arg
580 585 590
590

SEQ ID NO 4
LENGTH: 592
TYPE: PRT
ORGANISM: Artificial sequence
FEATURE:
OTHER INFORMATION: sequence coding for NS5b

SEQUENCE: 4
Met Ser Met Ser Tyr Thr Trp Thr Gly Ala Leu Ile Thr Pro Cys Ala
1 5 10 15
Ala Glu Glu Ser Lys Leu Pro Ile Asn Pro Leu Ser Asn Ser Leu
20 25 30
Arg His His Ser Met Val Tyr Ser Thr Thr Arg Ser Ala Ser Leu
35 40 45
Glu Arg Leu His Gly Leu Ser Ala Phe Ser Leu His Ser Tyr Ser Pro
465 470 475 480
Gly Glu Ile Asn Arg Val Ala Ser Cys Leu Arg Lys Leu Gly Val Pro
485 490 495
Pro Leu Arg Val Trp Arg His Arg Ala Arg Ser Val Arg Ala Lys Leu
500 505 510
Leu Ser Glu Gly Gly Arg Ala Ala Thr Cys Gly Lys Tyr Leu Phe Asn
515 520 525
Trp Ala Val Arg Thr Lys Leu Leu Thr Pro Ile Pro Ala Ala Ser
530 535 540
Gln Leu Asp Leu Ser Gly Trp Phe Val Ala Gly Tyr Asn Gly Gly Asp
545 550 555 560
Ile Tyr His Ser Leu Ser Arg Ala Arg Pro Arg Trp Phe Met Leu Cys
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590 595 599

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<213> ORGANISM: Artificial sequence
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5
96
ttg act gac ttc aag acc tgg ctc cag tcc aag ctc ctg ccg aaa ttg 96
Leu Thr Asp Phe Lys Thr Trp Leu Ser Lys Leu Leu Pro Lys Leu
20 25 30
144
ccg gga gtc cct ttc ttc tca tgc caa cgc ggg tac aag gga gtc tgg 144
Pro Gly Val Pro Phe Pro Phe Ser Cys Gln Arg Gly Tyr Val Gly Val Tryp
35 40 45
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cgg ggg gac ggc atg cta acc acc tgg oca tgg gga gaa csa att
Thr Gly Asp Gly Ile Met Gly Thr Thr Cys Pro Cys Gly Ala Gly Ile
50 55 60
240
acc gga cgt gtc aas acc acc tgg att ggg cct aas acc
Thr Gly His Val Lys Asp Gly Ser Arg Lys Gly Val Gly Thr Thr
65 70 75 80
288
tgc acc acc ggg cca aag tgg cac gag gac ttc acc aac acc aca
Cys Ser Asp Asp Gly Pro Gly Asp Asp Lys Asg Cys Thr Thr
85 90 95
336
ggc ccc tgc aca ccc tgg cgg cgg acc tat tcc agg ggg ctg tgg
Gly Pro Cys Thr Pro Ser Pro Ala Pro Asn Ser Arg Ala Leu Tryp
100 105 110
384
cgg gtt gct gct gaa gag cag tgg gac agg cgg ggc gcc 115
Arg Val Ala Ala Gly Thr Val Glu Ile Thr Arg Val Gly Asp Phe
115 120 125
432
cac tac gtt ctc ggc ata gaa cag tgg ggg cgg gaa cgg tgg cag
His Tyr Val Gly Met Thr Thr Asp Asn Val Lys Cys Pro Cys Gln
130 135 140
480
gtc cgg goc ccc gaa ttc act gaa tgt gac ggg tgt cgg tgt cac
Val Pro Ala Pro Glu Phe Phe Thr Glu Leu Asp Gly Val Arg Leu His
145 150 155 160

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528

Gln Val Gly Leu Aan Gln Tyr Leu Val Gly Ser Gin Leu Pro Cys Glu
180 185 190

Cct Cag Ccg Cag Gct Gag Gca Gct Gtc Act Too Atg Cto Acc Gac Ccc Too
624

Pro Glu Asp Val Ala Val Leu Thr Ser Met Leu Thr Asp Pro Ser
195 200 205

Cc Taa Cta Gct Aag Ctt Cag Cgg Ctc Tgc Ctc Ttc Gac Tcc Ccc Ctt
672

His Ile Thr Ala Glu Thr Asp Ala Arg Gly Ser Pro
210 215 220

Cc Ttc Cgg Tgc Tct Ctc Gag Cta Caa Ttc Ccg Ctc Gct Ctc Cca Tcc
720

Pro Ser Leu Ala Ser Ser Ala Ser Gin Leu Ser Ala Pro Ser Leu
225 230 235 240

Aag Cga Ctc Act Acc Ccc Ctt Gac Too Ccg Gac Gct Gac Ctc Ato
768

Lys Ala Thr Cys Thr Thr Pro Ala Asp Ala Pro Leu Ile
245 250 255

Gag Gcc Acc Ctc Ttg Cgg Cag Gct Atg Ggc Gga Aan Ile Thr Arg
816

Glu Ala Asn Leu Leu Trp Arg Glu Met Gln Gly Asn Ile Thr Arg
260 265 270

Gtg Gag Tca Gag Aat Aag Gtt Aag Gca Aag Gca Ggt Ctc Tcc
864

Val Glu Ser Glu Gln Val Val Leu Asp Ser Phe Asp Leu
275 280 285

Gca Gog Gaa Gag Gct Gag Aaa Gta Tcc Gga Gaa Gta Gca Gaa Gct Ctg
912

Arg Ala Glu Asp Gnu Val Val Ala Glu Ile Leu
290 295 300

Cga Csa Tcc Aag Tgc Tcc Cgg Gtc Caa Gtt Gca Gca Cct Tcc Tcc
960

Arg Lys Ser Lys Asp Pro Pro Leu Pro Ala Asp Pro Leu
305 310 315 320

Gat Tac Acc Cct Cgg Gcg Taa Tac Gag Gta Tac Gag Gta Tac
1008

Aep Tyr Aep Pro Pro Leu Leu Gln Ser Thr Pro Aep Tyr Val
325 330 335

Ccc Ccc Ctt Tgc Tct Gct Gag Gct Tct Aag Act Tgc Gct Ggc
1056

Pro Pro Ala Val His Cys Pro Pro Cys Pro Pro Thr
340 345 350

Ape Cgg Tct Tct Gcc Tct Gag Gcg Tct Gct Ctt Ctt Cgc Gct Ttc Cgc
1104

Ile Pro Pro Pro Arg Lys Arg Thr Val Val Leu Thr Glu Ser Thr
355 360 365

1152

Val Ser Ser Ala Ala Ala Leu Ala Thr Lys Thr Phe Gly Ser Ser
370 375 380

1200

Gly Ser Ser Arg Thr Ala Thr Ala Thr Ala Thr Ala Asp Pro
385 390 395 400

Acc Tct Gac Ggt Gct Gaa Aaa Gct Ttt Aaa Gtt Gta Tac Ctc Ctc
1248

Thr Ser Asp Asp Gnu Asp Pro Leu Thr Ser Thr Thr Thr Ser Ser
405 410 415

1296

Met Pro Pro Leu Gln Glu Glu Pro Gly Asp Pro Thr Ser Asp
420 425 430

Tct Ccc Tct Ctt Ctg Cgg Gag Ggc Gac Gac Gac Ctc Ggc Ggc Ggc Ggc Ggc
1344

Ser Thr Thr Val Ser Gly Ala Asp Ile Val Cys Cys
435 440 445
SEQUENCE: 6

Met Ser Gly Ser Trp Leu Arg Asp Val Trp Aep Trp Ile Cys Thr Val
1  5  10  15

Leu Thr Aep Phe Lys Thr Trp Leu Gln Ser Lys Leu Leu Pro Lys Leu
20 25 30

Pro Gly Val Pro Phe Phe Ser Cys Gln Arg Gly Tyr Lys Gly Val Trp
35 40 45

Arg Gly Aep Gly Ile Met Gln Thr Thr Cys Pro Cys Gly Ala Gln Ile
50 55 60

Thr Gly His Val Lys Asn Gly Ser Met Arg Ile Val Gly Pro Lys Thr
65 70 75 80

Cys Ser Asn Thr Trp His Gly Thr Phe Pro Ile Asn Ala Tyr Thr Thr
85 90 95

Gly Pro Cys Thr Pro Ser Pro Ala Pro Asn Tyr Ser Arg Ala Leu Trp
100 105 110

Arg Val Ala Ala Glu Glu Tyr Val Glu Ile Thr Arg Val Gly Asp Phe
115 120 125

His Tyr Val Thr Gly Met Thr Thr Asp Asn Val Lys Cys Pro Cys Gln
130 135 140

Val Pro Ala Pro Glu Phe Phe Thr Glu Leu Asp Gly Val Arg Val Arg His
145 150 155 160

Arg Tyr Ala Pro Ala Cys Arg Pro Leu Leu Arg Val Asp Val Thr Phe
165 170 175

Gln Val Gly Leu Asn Gln Tyr Leu Val Gly Ser Gln Leu Pro Cys Glu
180 185 190

Pro Glu Pro Asp Val Ala Val Leu Thr Ser Met Leu Thr Asp Pro Ser
195 200 205

His Ile Thr Ala Glu Thr Thr Lys Arg Arg Pro Ala Arg Gly Ser Pro
210 215 220

Pro Ser Leu Ala Ser Ser Ser Ala Ser Gln Leu Ser Ala Pro Ser Leu
225 230 235 240

Lys Ala Thr Cys Thr Thr His His Asp Ser Pro Ala Asp Ala Asp Leu Ile
245 250 255

Glu Ala Asn Leu Leu Leu TrpArg Gln Glu Met Gly Asn Ile Thr Arg
260 265 270

Val Glu Ser Asn Lys Val Val Leu Asp Ser Phe Asp Pro Leu
275 280 285

Arg Ala Glu Asp Glu Arg Val Ser Val Ala Ala Glu Ile Leu
290 295 300

Arg Lys Ser Lys Lys Phe Pro Ala Leu Pro Ile Trp Ala Arg Pro
305 310 315 320

Asp Tyr Asn Pro Pro Leu Leu Glu Ser Trp Lys Ser Pro Asp Tyr Val
325 330 335

Pro Pro Ala Val His Gly Cys Pro Leu Pro Pro Thr Thr Gly Pro Pro
340 345 350

Ile Pro Pro Pro Arg Lys Lys Arg Thr Val Val Leu Thr Glu Ser Thr
Val Ser Ser Ala Leu Ala Glu Leu Ala Thr Lys Thr Phe Gly Ser Ser
370  375  380
Gly Ser Ser Ala Val Asp Ser Gly Thr Ala Thr Ala Pro Pro Aep Gln
385  390  395  400
Thr Ser Asp Asp Gly Asp Lys Ser Asp Ile Glu Ser Tyr Ser Ser
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420  425  430
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<211> LENGTH: 2241
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Arg Thr Asn
   14 15 16 17 18 19

   20 21 22
Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg

   23 24 25 26 27 28 29
Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg

   30 31 32 33 34 35
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   36 37 38 39 40 41 42
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aac tca aat att gtg tat gag gca gcg gao atg atc atg cac accccc 210 215 220

Gly Cys Val Pro Cys Val Arg Glu Ala Aap Met Ile Met His Thr Pro 220 225 230

ggg tgc tgtc tgc cgg gaq gat ttt ttc cct gtt gtc tgtg gta 235 240 720

gcg ctg act ccc aqc ctg cgc gac aac aqc gtc ccc acc acc 245 250 255

Asl Atp Thr Pro Thr Leu Ala Aap Aen Ser Ser Ile Pro Thr Thr 260 265 270

aca ata cga cgc cac gtc get ttc gtc tgg ctc gcc ctg ttc gcg gat tgc gtc gta caa gat tgc gtc get ttc 275 280 285 816

Thr Ile Arg Arg His Val Aen Phe Leu Leu Leu Val Ser Cys Gln 290 295 300

cag gtg ctc acc ttc tai cct cgc tgg tat gca gcg gac atg atc 300 864

Thr Leu Phe Phe Thr Phe Ser Pro Thr Ile Val Thr Val Gln 305 310 315

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Asn Ser Tyr Val Glu Val Phe Ser Ser Thr Gln Ser Gly 340 345 350

tgc gca gtc tgg cct gag ttc cct cag gun agc atc ttc ggt tat tcc 355 360 365

Ser Ala Met Tyr Val Gly Ala Ala Tyr Ser Met Val Glu Aen Aap Cys 370 375 380

acedt cgg ttc acc tat gca ggc cgg tag agc gta caa gat tgc gtc gta 390 395 400

Val Leu Val Leu Ser Gly Thr Gly Thr Val Thr Val Gln 405 410 415

acc aac tgg cca ggg ggt gca gtc tgg gag gtc aac gag ctc tgt 420 425 430 1104

Thr His Thr Tyr Val Glu Arg Val Aap Thr Ser Thr Gin Ser Leu Val 435 440 445

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Arg Ser Aap Met Thr Gln Gly Ser Phe Leu Ser Gly Leu Ser Gly Thr 470 475 480

gac cag agg ctc tgc ctc ctc ctc ctc aac aac ctc gtc gtc gtc gtc 495 500 505

Asp Glu Arg Pro Tyr Cys Trp His Tyr Ala Pro Pro Cys Gly Gly 510 515 520
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ccg gtt gtt gtt ggg acg acc gac ccg ccg ggc acc ccg aat ggc Pro Val Val Val Gly Thr Asp Gly Ser Gly Val Pro Thr Tyr Ser 500 526 525 1584
tgg ggg gag att gaa gag cag gtt ctg ctc ttc tac acc aac ccg ggc Trp Gly Glu Asn Glu Thr Aep Val Leu Leu Asn Thr Arg Pro 530 540 1632

ccg ccc gcg tgg ccc tgg ggc ccc cgg ggc ccc ggc ccc ggc tgg Pro Gln Gly Asn Trp Phe Gly Cys Thr Tct Met Asn Ser Thr Gly Phe 545 555 560 1680

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acc acc ttc att tgc ccc acg gat ggc ccc tgg ctc ctt ctt ctc ctg Aem Thr Leu Ile Cys Pro Thr Asp Cys Pro Cys Asp Gln Gly Ser 590 595 599 1776

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gtt gac tac cca tac aga ctt ccc ttc tcc cca tcc acc act acc ctc Val Asp Tyr Pro Tyr Arg Leu Trp His Tyr Pro Cys Asp Thr Val Asp 630 635 640 1872

acc acc ccg aga ggc cgg ggc ggc ggc ggc ggc ggc ggc ggc ggc Thr Ile Phe Lys Val Met Tyr Val Gly Gly His Arg Leu 645 650 655 1920

acc ggc ggc tgc att acc cga gga gag ccg tgc atg gag cag gag ggc Aem Ala Ala Cys Aem Trp Thr Ala Cys Asp Gln Gly Glu Ala 665 670 675 1968

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gta ggg gca gtt gtt gct gcc cga aac cgg ggt tgg cgg ggc aat Val Gly Ser Val Val Ser Val Val Leu Lys Trp Gly Tyr Leu Leu 740 745 2160

ctg ctc ctc ctc ctc ctc ggc gac ggc ggc ggc ggc ggc ggc ggc Leu Leu Leu Leu Leu Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Leu 765 770 775 2208

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1. A method of treatment of one or more pathologies associated with an hepatitis C virus, which comprises at least one administration to a host organism of an effective dose of a peptide composition characterized in that said composition comprises a polyprotein NS3/NS4 of an hepatitis C virus, as well as a polypeptide NS5b of an hepatitis C virus.

2. A method of stimulating a T cell-mediated immune response against an hepatitis C virus target protein which comprises the step of administering in a host organism at least one dose of a peptide composition characterized in that said peptide composition comprises a polyprotein NS3/NS4 of an hepatitis C virus, as well as a polypeptide NS5b of an hepatitis C virus, so as to stimulate a host's T cell-mediated immune response.

3. The method according to claim 1 or 2, wherein NS3 and/or NS4 and/or NS5b originate from viruses of different genotypes.

4. The method according to claim 1 or 2, wherein NS3, NS4 and NS5b originate from a virus of the same genotype, preferably genotype 1b.

5. The method according to claim 1 or 2, wherein the NS3/NS4 polyprotein comprised in the peptide composition originates from a genotype 1b and the hepatitis C virus associated to the pathologies to be treated or to the target protein is of genotype 1a.

6. The method according to claim 1 or 2, wherein said peptide composition is in association with a pharmaceutically appropriate vehicle.

7. The method according to claim 1 or 2, wherein said method comprises at least three sequential administrations of said peptide composition, which are independently separated by a period of time varying from 3 days to 10 days.

8. The method according to claim 7, wherein said method further includes at least one “recall” administration at the end of the at least three sequential administrations.

9. The method according to claim 2, wherein said stimulated immune response is a CD8+ T cell response, a CD4+ T cell response or both CD8+ and CD4+ T cell responses.

10. A method of treatment of one or more pathologies associated with an hepatitis C virus, which comprises at least one administration to a host organism of an effective dose of an expression vector characterized in that said expression vector comprises a nucleotide sequence coding for a polyprotein NS3/NS4 of an hepatitis C virus and a nucleotide sequence coding for a polypeptide NS5b of an hepatitis C virus, as well as the means necessary to their expression.
11. A method of stimulating a T cell-mediated immune response against an hepatitis C virus target protein which comprises the step of administering in a host organism at least one dose of an expression vector characterized in that said expression vector comprises a nucleotide sequence coding for a polyprotein NS3/NS4 of an hepatitis C virus and a nucleotide sequence coding for a polypeptide NS5b of an hepatitis C virus, as well as the means necessary to their expression, so as to stimulate a host’s T cell-mediated immune response.

12. The method according to claim 10 or 11, wherein said nucleotide sequences code for a polyprotein and a polypeptide originating from viruses of different genotypes.

13. The method according to claim 10 or 11, wherein said nucleotide sequences code for a polyprotein and a polypeptide originating from a virus of the same genotype, preferably the genotype 1b.

14. The method according to claim 10 or 11, wherein the NS3/NS4 polyprotein encoded by the expression vector originates from a genotype 1b and the hepatitis C virus associated to the pathologies to be treated or to the target protein is of genotype 1a.

15. The method according to claim 10 or 11, wherein the expression vector is an adenovirus.

16. The method according to claim 10 or 11, wherein the expression vector is a poxvirus.

17. The method according to claim 16, wherein the genome of the adenovirus is modified so as to replace the EI region by the expression cassette CMV-NS3-NS4 and to replace the E3 region by the expression cassette SV40-NS5b.

18. The method according to claim 10 or 11, wherein the expression vector is a poxvirus.

19. The method according to claim 18, wherein the genome of the poxvirus is modified so as to insert the expression cassette ph5r-NS3-NS4 and to insert the expression cassette p7.5-NS5b.

20. The method according to claim 18, wherein the expression vector is a MVA whose genome is modified so as to insert the expression cassette ph5r-NS3-NS4 and the expression cassette p7.5-NS5b in deletion III and in the same direction.

21. The method according to claim 10 or 11, wherein said method comprises at least three sequential administrations of said expression vector, which are independently separated by a period of time varying from 3 days to 10 days.

22. The method according to claim 21, wherein said method comprises three sequential administrations at approximately one week interval of the expression vector and wherein said expression vector is administered to the host organism by intramuscular route.

23. The method according to claim 22, wherein said method further includes at least one “recall” administration at the end of the at least three sequential administrations.

24. The method according to claim 23, wherein said method comprises one recall administration which takes place at least approximately 4 weeks after the latest of the at least three sequential administrations.

25. The method according to claim 23, wherein said method comprises one recall administration which takes place approximately 6 months after the latest of the at least three sequential administrations.

26. The method according to claim 1 or 2, wherein the method is carried out in conjunction with treatment of the host organism with pegylated IFN-α2 and/or ribavirin.

27. The method according to claim 10 or 11, wherein the method is carried out in conjunction with treatment of the host organism with pegylated IFN-α2 and/or ribavirin.

28. The method according to claim 11, wherein said stimulated immune response is a CD8+ T cell response, a CD4+ T cell response or both CD8+ and CD4+ T cell responses.

29. The method according to claim 11, wherein said hepatitis C virus target protein is an epitope located in a NS3, and/or a NS4 and/or a NS5b protein.

30. The method according to claim 29, wherein said epitope is HLA-B7-restricted epitope and is located in the NS3 polypeptide of the infecting hepatitis virus.

31. The method according to claim 29, wherein said epitope is HLA-A2-restricted epitope and is located in the NS3 and/or NS5b protein of the infecting hepatitis virus.