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(54) **HUMAN MONOCLONAL ANTIBODY FAB FRAGMENTS DIRECTED AGAINST HCV E2 GLYCOPROTEIN AND ENDOWED WITH IN VITRO NEUTRALIZING ACTIVITY**

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(57) **ABSTRACT**

The invention refers to a human antibody, or its functional fragments, directed against the HCV E2 glycoprotein, able to have a neutralizing activity in vivo; a composition for anti-HCV therapy comprising in a therapeutically effective amount the antibody; a composition for topical use in gel, creme, ointment and ovule formulations; the use of the antibody for validating anti-HCV vaccines.

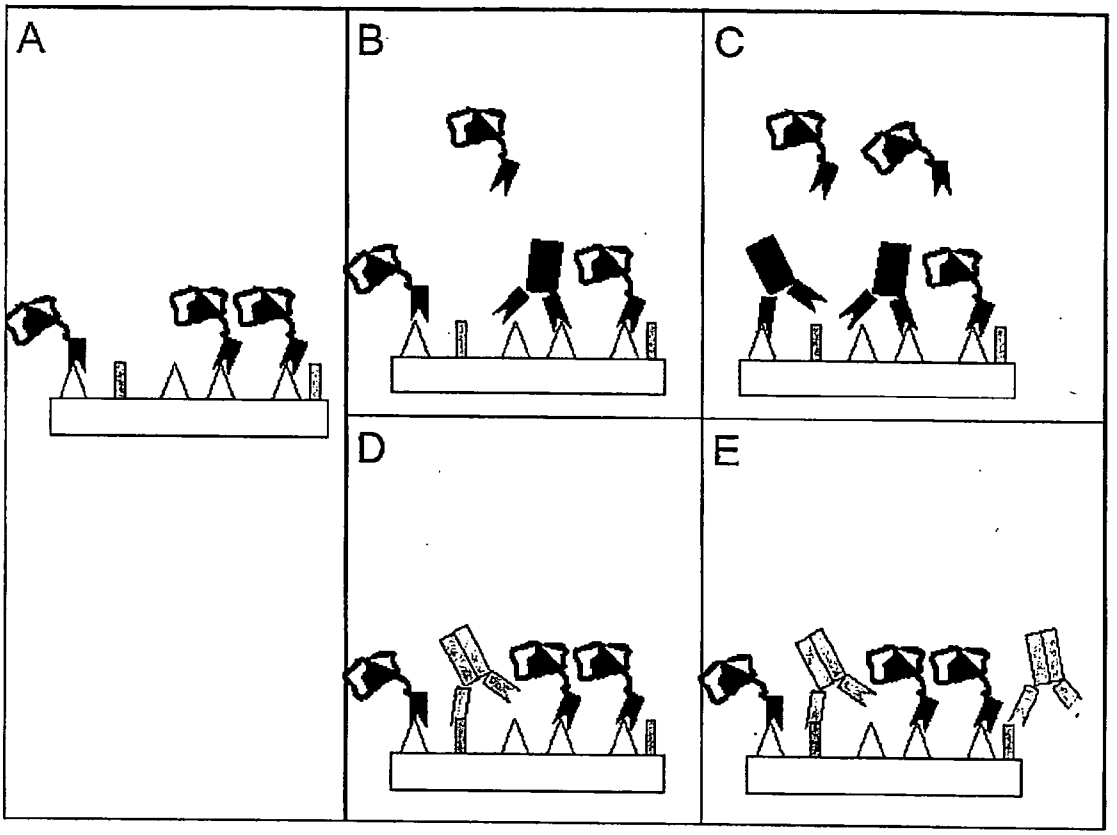

FIGURE 1

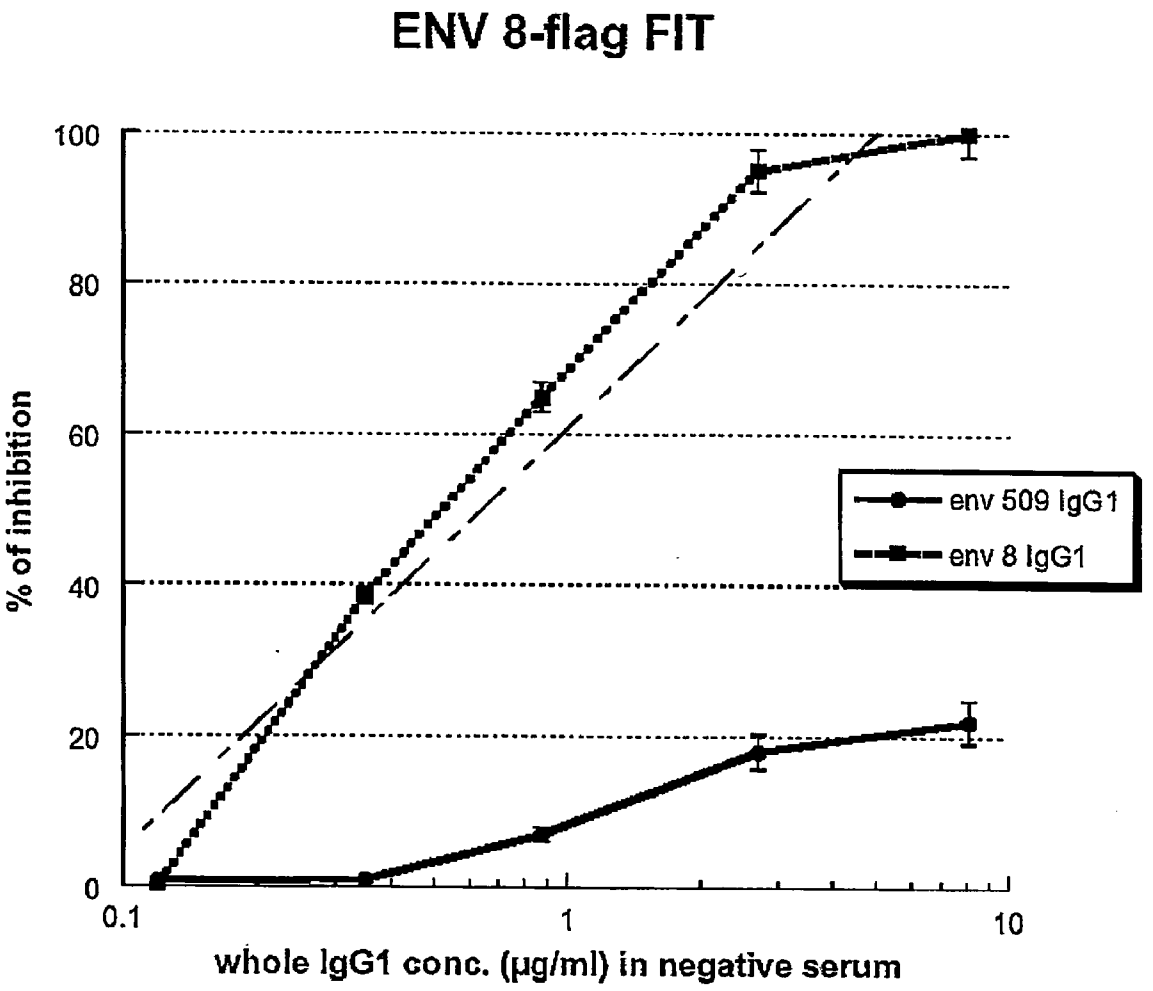
ENV 8-flag FIT
100
80
60
40
20
0
% of inhibition
0.1
1
10
whole IgG1 conc. (µg/ml) in negative serum
env 509 IgG1
env 8 IgG1

FIGURE 2A

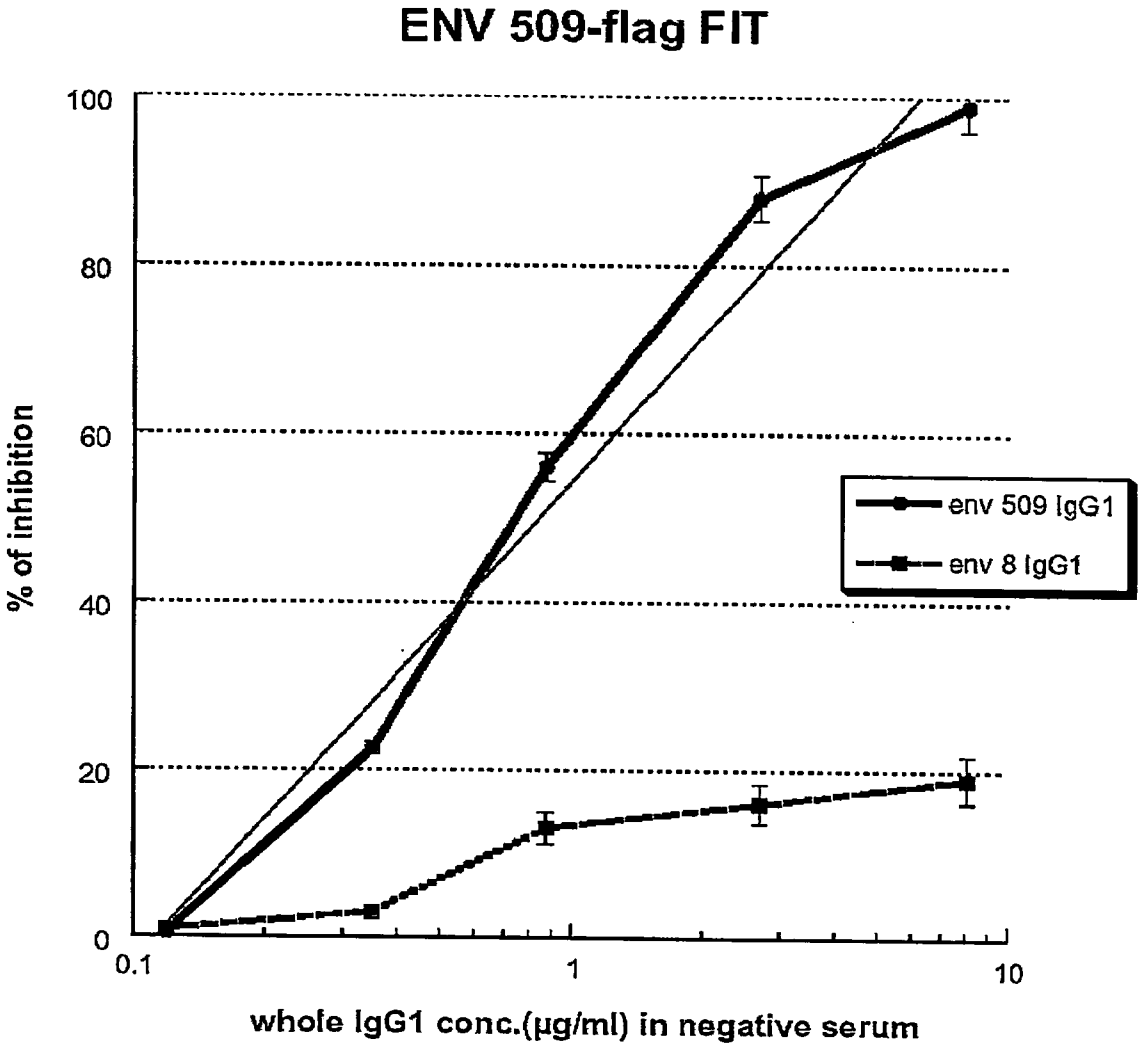
ENV 509-flag FIT
100
80
60
40
20
0
% of inhibition
0.1
1
10
whole IgG1 conc.(µg/ml) in negative serum
env 509 IgG1
env 8 IgG1

FIGURE 2B

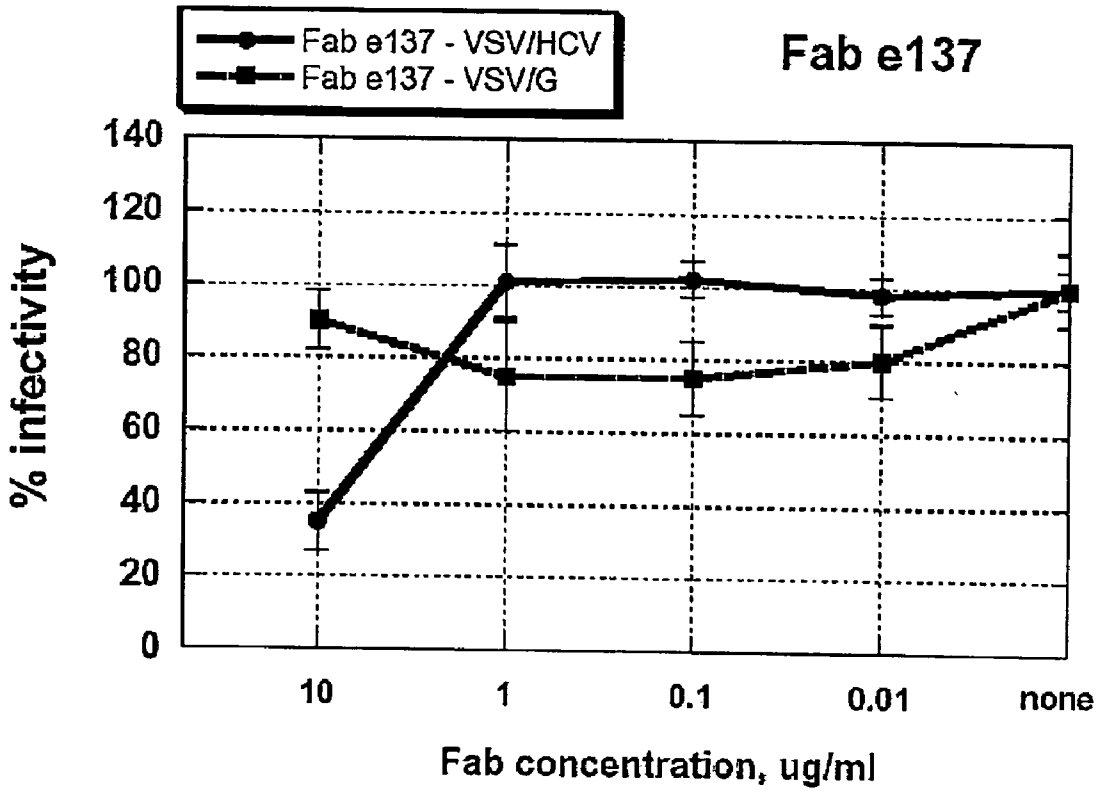
Fab e137 - VSV/HCV
Fab e137 - VSV/G
Fab e137
% infectivity
140
120
100
80
60
40
20
0
10
1
0.1
0.01
none
Fab concentration, ug/ml

FIGURE 3A

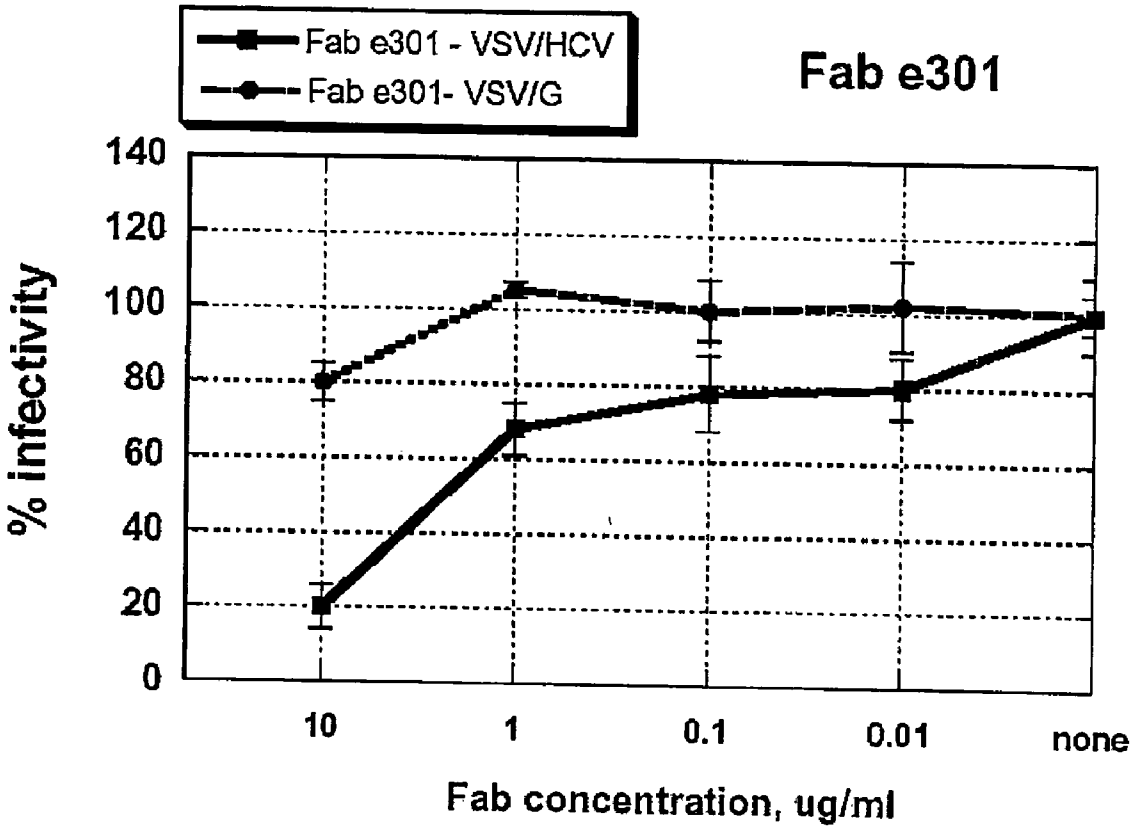
Fab e301 - VSV/HCV
Fab e301- VSV/G
Fab e301
% infectivity
140
120
100
80
60
40
20
0
10
1
0.1
0.01
none
Fab concentration, ug/ml

FIGURE 3B

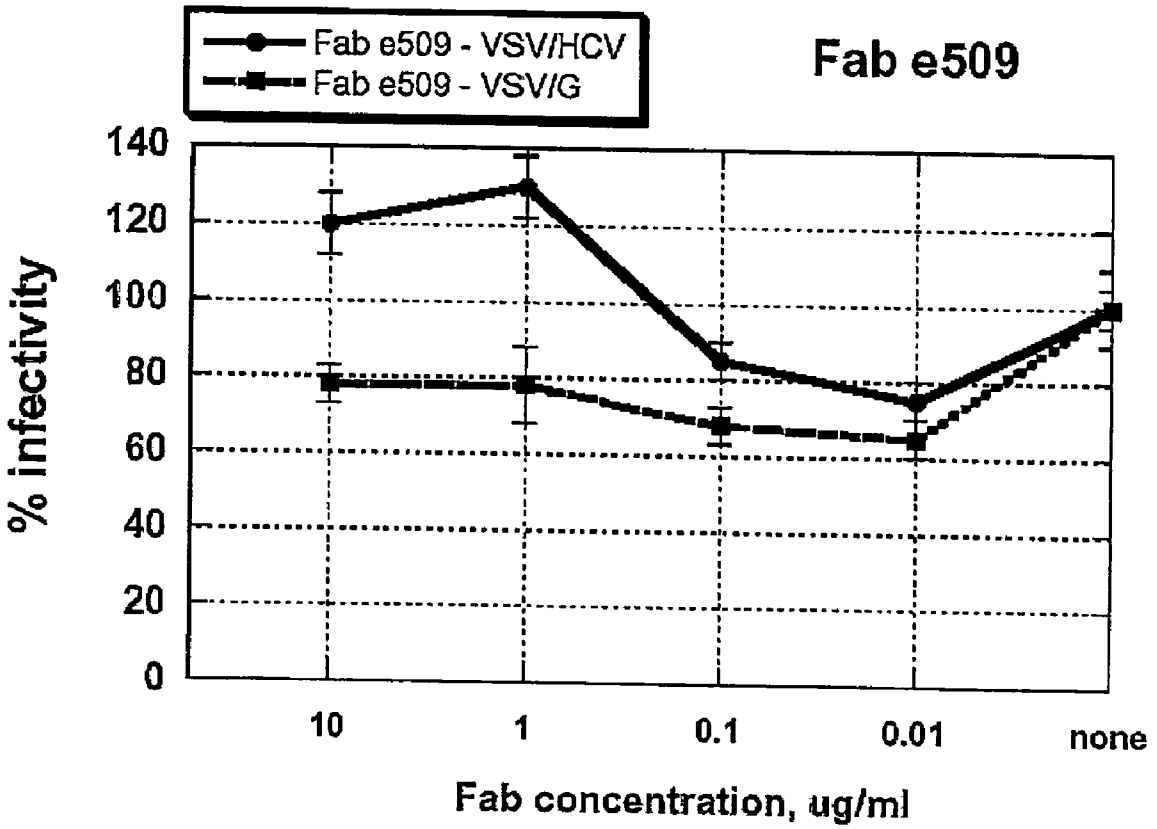
Fab e509 - VSV/HCV
Fab e509 - VSV/G
Fab e509
% infectivity
140
120
100
80
60
40
20
0
10
1
0.1
0.01
none
Fab concentration, ug/ml

FIGURE 3C

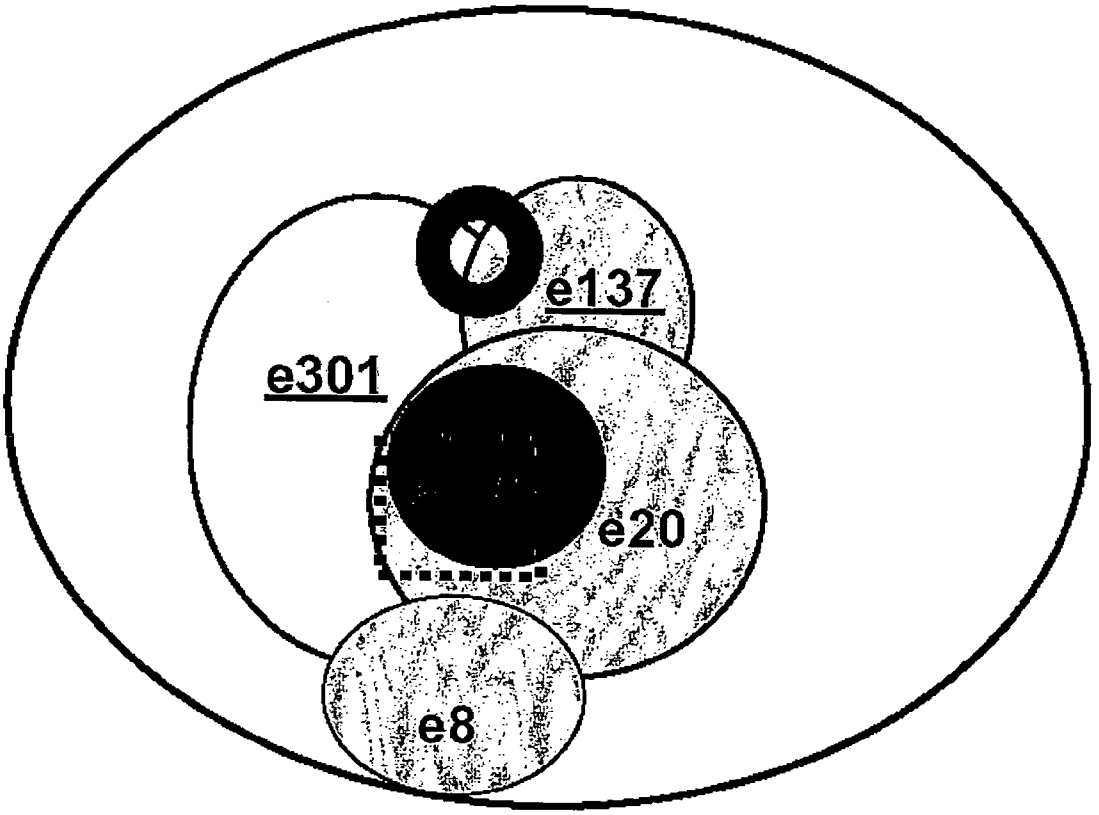

FIGURE 4

HUMAN MONOCLONAL ANTIBODY FAB FRAGMENTS DIRECTED AGAINST HCV E2 GLYCOPROTEIN AND ENDOWED WITH IN VITRO NEUTRALIZING ACTIVITY

[0001] The invention concerns human monoclonal antibody Fab fragments directed against HCV E2 glycoprotein and endowed with in vitro neutralizing activity. Hepatitis C virus (VCV) infects about 4% of the world population (World Health Organization, 1999). Over 80% of subjects coming into contact with this pathogen develop a chronic infection as the host immune response is unable to eradicate the infection, with the risk of severe liver diseases such as chronic hepatitis, cirrhosis and liver cell carcinoma [1, 2].

[0002] Treatment of chronic infection is based on combined therapy with interferon and ribavirin, which is extremely costly causes major side effects and is moderately effective (only 1 patient in 4 obtains long-term results) [3, 4]. The viral infection does not provide immune protection. This fact, together with the virus's high variability in antigenic structure recognized by the immune system, has hindered the development of an effective serum therapy and vaccines to protect individuals against HCV infection. It is therefore clear that new antiviral strategies are strongly needed.

[0003] The author has cloned the genes coding for a large number of human Fabs antibody fragments directed against one of the HCV proteins, the external E2 glycoprotein, considered the most important target for immune protective response [5]. However, the evaluation of the biological activity of these antibody fragments is not simple, as no reliable in vitro systems are available to determine the neutralizing activity against HCV. Hence, the author has only evaluated and described the variable ability of different Fabs to inhibit the binding of protein E2 to the target cell, without demonstrating a correlation between this activity and the neutralizing activity of the sera [5].

[0004] In a previous work, Burioni et al. (2001) [6], showed that some anti-E2 antibodies produced by HCV-infected patients have a negative effect, rendering the virus less sensitive to host immune response, probably due to its binding to the E2 antigen and to modifications of its conformation [6]. This could explain why high anti-E2 antibody titers are not directly correlated with protection against HCV infection.

[0005] Bugli et al., 2001 [7] generated a map of E2 protein epitopes that can bind in vitro the panel of anti-E2 human Fabs, showing four discrete regions against which immune response is directed (**FIG. 2**) [7]. The presence of antibodies directed against one or more of these regions in the serum of chronically infected patients could be associated with complications, reduced effectiveness of treatment and a different prognosis. It is therefore evident that there is a need for a method to determine antibodies in a biological fluid directed against different epitopes of the HCV E2 protein. An embodiment of the present invention provides this method.

[0006] The authors of the invention have also evaluated the neutralizing activity of various anti-E2 antibodies in a system of viral pseudotypes, i.e. viruses externally identical to HCV but, after entering the target cells which are able to produce a protein that produces fluorescence [8]. By revealing the presence or absence of fluorescence in the cells, the method provides a direct measure of the in vivo neutralizing activity of anti-E2 antibodies directed against different epitopes.

[0007] Unexpectedly, the authors found that two of the assayed antibodies, e137 and e301, can neutralize the virus at concentrations obtainable with a single parenteral administration of an antibody preparation; two other antibodies had no neutralizing activity and one was even able to promote viral infection.

[0008] The development of the method of titering different antibody populations in a patient represents a valuable diagnostic and prognostic instrument with the potential to distinguish between affected subjects at risk for developing severe complications and those with a more favorable prognosis. In this latter group, this method would eliminate the need to administer a largely ineffective treatment that is also associated with severe side effects, while providing a considerable reduction in costs.

[0009] As the E2 epitopes, so identified, are not reproducible by synthesizing synthetic peptides [5], the method represents the only way to determine the amount of antibodies against the different parts of the protein E2, with correlated clinical and epidemiological data.

[0010] The identification of anti-E2 antibodies in the human Fabs format with a good neutralizing ability permits their large-scale production and use as a medication in anti-HCV treatment, or as a preventive agent in topical form to inhibit viral transmission to subjects at risk (couples with discordant HCV state, individuals subject to occupational exposure, etc.).

[0011] The antibodies of the invention can be advantageously used to evaluate in vitro candidate molecules for anti-HCV vaccines, i.e. able to stimulate neutralizing antibodies but not ineffective or negative antibodies.

[0012] The availability of neutralizing human antibodies able to recognize a broad spectrum of viruses could be crucial in the production of artificial vaccines. The neutralizing antibodies described in this document can be used as a template for the development of vaccines (made from peptides or anti-idiotype antibodies) able to stimulate a neutralizing cross-reactive response.

[0013] The object of this invention is a human antibody, or its functional fragments, against the HCV E2 protein, endowed with an in vivo neutralizing activity.

[0014] In a particular embodiment, the antibody of the invention is the antibody e137, which is characterized by the following amino acid sequences of the variable part of the heavy and light chains:

e 137 Heavy chain (HC)

LLEQSGSEVKVPGSSLKVSCKTSGGTFSTYTFSWVRQAPGQGLEWMG

GITPIIGIANYARNFQDRVTITADESTSTVYMEVRRLRSEDTAVYYCAKTS

EVTATRGRTFFYSAMDVWGQGT

-continued

e 137 Light chain (LC)

MAELTQSPSFLSASVGDRVTITCRASQGISNYLAWYQQKPGKAPKLLIYA

ASTLQSGVPSRFSGSGSWTEFTLTISRLQPEDFATYYCQHLNTYPWTFG

QGT

[0015] In an alternative embodiment, the antibody of the invention is the antibody e301, which is characterized by the following amino acid sequences of the variable part of the heavy and light chains:

e 301 Heavy chain (HC)

LLEQSGSEVKKPGSSVRVSCTTSGGTLSDYGFNWLRQAPGQGPEWMG

GIIPLFRRTTYGQKFQGRLTITADESTGATYMELSSLRSDDTAVYYCARE

KVSVLTGGKSLHYFEYWGKGT

e 301 Light chain (LC)

MAELTQSPATLSVSPGERATLSCRASQSVSSRLAWYQQKRGQAPSLLIY

DTSSRATGVPARFSASGSGTQFTLTISSLQSEDFALYYCQQYNDWPSTF

GQGT

[0016] A further object of the invention is a composition for anti-HCV therapy comprising in a therapeutically effective amount at least one of the antibodies of the invention. Preferably, the composition is supplied in purified form for parenteral use or in another formulation for topical use as a gel, creme, ointment, ovule, with excipients known to experts in the field. A further object of the invention is a nucleic acid coding for each of the antibodies of the invention. Advantageously, the nucleic acid can be contained in an expression vector which can effectively express the antibody of the invention in prokaryote or also in eukaryote cells. In a preferred form, the recombinant vector also contains a nucleotide sequence coding for a signal peptide which is substantially contiguous with the coding sequence for the antibody of the invention, and is able to export the antibody out of the cell environment.

[0017] A further object of the invention is the use of the recombinant vector as described in gene therapy.

[0018] The invention is described below in experimental examples, not limiting the invention itself, in reference to the following figures:

[0019] **FIG. 1** FIT: THEORETICAL BASIS. Panel A shows the binding of a Fab-FLAG to its epitopes without competitors. Using the same concentration of Fab present in (A), preincubation of the antigen with the patient's serum permits quantitative analysis of antibodies directed against the epitope recognized by the Fab in the serum. In panels B and C, the bound antibodies. as they compete with Fab, proportionately diminish the amount bound compared with panel A. In panels D and E, the presence of antibodies not directed against the specific epitope does not minimally influence Fab binding.

[0020] **FIG. 2 A** and B: Inhibition of binding between e8-FLAG (A) and e509-FLAG (B) to HCV/E2 by sera containing known concentrations of e8-IgG1 and e509-IgG1 (whole antibodies directed against the epitopes recognized by the Fab). It is clear that the inhibition of Fab binding can be observed only in the presence of the whole antibody having the same specificity and that this depends on antibody concentration.

[0021] **FIGS. 3A**, B and C: Inhibition of infection of VSV/HCV and VSV/G pseudotypes by purified anti-HCV/E2 human recombinant Fabs at different concentrations. HepG2 cells infected with Fab-treated pseudotypes were incubated for 16 hr and the number. of green fluorescent protein-expressing cells was determined by fluorescence microscopy. Data are presented as % of the infection detected in control wells (no Fabs added). The results shown are the average of three independent assays performed in double.

[0022] **FIG. 4**: Two-dimensional surface-like map of the human B cell epitopes present on the surface of HCV/E2 as recognized by the monoclonal antibodies used in this study. Overlapping circles indicate reciprocal inhibition. Fabs endowed with VSV/HCV pseudotype neutralizing activity are underlined. The putative region mediating the interaction of HCV/E2 with the cellular target is indicated by the dotted line. The putative region recognized by neutralizing antibodies is indicated by a solid black circle. Due to modifications that can be induced by antigen-antibody interactions, this diagram does not correspond to the actual physical map.

EXAMPLE 1

[0023] Materials and Methods

[0024] Anti-HCV Fabs and Full-Size IqG1 Production

[0025] Generation, purification and characterization of the anti-HCV/E2 Fabs have been described elsewhere [5]. FLAG-Fabs (Fabs labeled with a FLAG epitope fused at the carboxyterminal of the heavy chain fragment with a pentapeptide bridge) were constructed and purified as described elsewhere [6]. Validation and standardization of the assay were performed using Fab-coding genes to construct full-size human monoclonal antibodies (HuMabs), which were inserted in an appropriate eukaryotic vector for subsequent production in transfected cells [9]. The HuMabs present in the culture supematant were purified by immunoaffinity as described [10] and purity-checked by PAGE. The amount of human antibody was assayed by a sandwich immunoassay. All antibodies and Fabs were stored at −70° C. until use.

[0026] Sera and Specimens

[0027] Sera obtained from healthy donors and HCV-positive patients were analyzed using commercial diagnostic kits (Ortho, Raritan, N.J.) following standard procedures. For the preparation of mock specimens with known amounts of antibodies directed against a given epitope, HCV-negative sera were spiked with concentrated purified HuMabs in PBS and treated exactly like the positive and negative sera.

[0028] Design of Fab Inhibition Titer (FIT) Assay

[0029] The purpose of this assay is to assess the ability of sera to inhibit the binding of a labeled Fab to its epitope, thus obtaining an indirect measure of the amount of epitope-binding antibodies in sera (**FIG. 1**).

[0030] FLAG-Fabs were purified [10] and assayed in a FLAG-Fab-specific ELISA to determine the correct concen-

tration to be used in inhibition experiments. Briefly, FLAG-Fab preparations of known concentration were titered by ELISA[11], where antigen-coated plates were blocked for 1 h at 37° C. with PBS/1% BSA. After removing the blocking solution, 50 μl of progressive dilutions of FLAG-Fab made in PBS/BSA 1% were added to the wells and incubated for 2 h at 37° C. Plates were washed 10 times with PBS/0.05% Tween-20 in an automated plate washer (DiaSorin, Saluggia, Italy) before adding 50 μl of a 10 μg/ml solution of anti-FLAG mouse monoclonal antibody M2 (Sigma, St. Louis, Mo.; 10 μg/ml in PBS) in PBS/BSA 1%. After 1 h incubation at 37° C., wells were washed 10 times with PBS/Tween-20 as above and mouse monoclonal antibody binding was revealed with horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce; 1:8,000 in PBS). Substrate was added and plates were read for OD_{450} in an automated plate reader after 30 min incubation at room temperature in the dark. All assays were performed at least in double. A negative control antigen (BSA) was always included and the OD reading was subtracted as background.

[0031] For the determination of the Fab Inhibiting Titer (FIT) of sera, a concentration of purified FLAG-Fabs yielding in standard conditions an OD_{450} reading equal to 50% of maximum reading was used for further experiments of Fab inhibition ELISA. For these experiments, plates were coated and blocked as described above. Progressive 1:4 serum dilutions in PBS/BSA 1% were added in the amount of 50 μl per ELISA well. After 2 h of incubation at 37° C., purified FLAG-Fab was added directly to serum dilutions to reach the desired final concentration. Plates were incubated for additional 30 min and then processed as described above for FLAG-Fab ELISA. A positive control sample, containing a 20:1 excess of purified unlabeled Fab, corresponding to 100% inhibition, is included. A negative control sample, containing an excess of a control uncorrelated Fab [12] and corresponding to 0% inhibition, is also included. The final results are determined as % of inhibition with the formula: percent inhibition=100×(OD_{450} of probe FLAG-Fab alone—OD_{450} of probe FLAG-Fab with competing serum)/OD_{450} of probe FLAG-Fab alone.

[0032] The highest serum dilution giving more than 70% inhibition of FLAG-Fab binding is considered as the Fab Inhibiting Titer (FIT) for that epitope and for that serum.

[0033] Results

[0034] The appropriate FLAG-Fab concentration to be employed in the assay is determined for each FLAG-Fab and ranges from 10 μg/ml (e8, e20, e137, e301, e509) to 0.1 μg/ml (e10-B). The amino acid sequences of the light and heavy chains of the various antibodies are given below:

e8 HC

LLEQSGAEVKMPGATVKVSCQSSRYTFTSYGIGWVRQAPGQGLEWMG

WISGYTHETKYAQSFQGRVTMTAETSTGTAYMELRSLRSDDTATYYCA

RDGGGRVVVPPTHLRAFDVWGQGT

e8 LC

MAELTQSPGTLSLSPGERATLSCRASHRVNNNFLAWYQQKPGQAPRLLI

SGASTRATGIPDRFSGSGSGTDFTLTISRLEPDDFAVYYCQQYGDSPLY

SFGQGT

-continued

e10 HC

LLESGPGLVKPSQTLSLTCTVSGVSISYGGRGVSYWGWVRQSPGKGLE

WIGHIYYFGDTFYNPSLNNRATISIDSSKNQFSLKLKSVTASDTALYFCAR

STLQYFDWLLTREAAYSIDFWGQGI

e10 LC

MAELTQSPSFLSASVGDRVTITCRASQGVTILLAWYQQKPGKPPKALIYA

ASSLQSGVPSRFSGSGSDTDFTLTISSLQPEDSATYYCQQLNTYPWTFG

QGT

e20 HC

LLEQSGAEVKKPGSSVKVSCKASGDHYGINWVRQAPGQGLEWMGGIIP

VFGTTTYAQKFQGRATITADDSTGTAFLELTRLTFDDTAVYFCATPHQLH

VLRGGKALSPWDYWGQGT

e20 LC

MAELTQSPATLSVSPGERATLSCRASQSVSSNLAWYQQKRGQAPSLLIY

GTSTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNDWPSTF

GQGT

e137 HC

LLEQSGSEVKVPGSSLKVSCKTSGGTFSTYTFSWVRQAPGQGLEWMG

GITPIIGIANYARNFQDRVTITADESTSTVYMEVRRLRSEDTAVYYCAKTS

EVTATRGRTFFYSAMDVWGQGT

e137 LC

MAELTQSPSFLSASVGDRVTITCRASQGISNYLAWYQQKPGKAPKLLIYA

ASTLQSGVPSRFSGSGSWTEFTLTISRLQPEDFATYYCQHLNTYPWTFG

QGT

e301 HC

LLEQSGSEVKKPGSSVRVSCTTSGGTLSDYGFNWLRQAPGQGPEWMG

GIIPLFRRTTYGQKFQGRLTITADESTGATYMELSSLRSDDTAVYYCARE

KVSVLTGGKSLHYFEYWGKGT

e301 LC

MAELTQSPATLSVSPGERATLSCRASQSVSSRLAWYQQKRGQAPSLLIY

DTSSRATGVPARFSASGSGTQFTLTISSLQSEDFALYYCQQYNDWPSTF

GQGT

e509 HC

LLEESGAEVKKPGSSVKVSCKTSGDTFRYGITWVRQAPGQGLEWMGQI

MPTFATATYAQRFQGRVTISADESTSTAYLEVRSLRSEDTAVYYCATPR

QVTILRGPKALSPWDYWGQGT

-continued

e509 LC

MAELTQSPATLSASPGERASLSCRASQSVSSNLAWYQQKPGQAPRLLIS

GASTRATGVPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWPPH

FGQGT

[0035] The nucleotide sequences coding for the Fab fragments listed above are indicated as follows:

e8 HC

CTGCTCGAGCAGTCTGGAGCTGAGGTGAAGATGCCTGGGGCCACAG

TGAAGGTCTCCTGCCAGTCTTCCCGTTACACCTTCACCAGTTACGGT

ATCGGCTGGGTGCGACAGGCCCCTGGACAGGGGCTTGAGTGGATG

GGATGGATCAGCGGATACACCCATGAGACAAAATATGCACAGAGTTT

CCAGGGCAGAGTCACCATGACCGCAGAGACATCCACGGGCACAGCG

TATATGGAGTTGAGGAGCCTGCGGTCTGACGACACGGCCACATATTA

CTGCGCGAGAGATGGAGGAGGGAGGGTGGTAGTGCCGCCTACTCAT

CTACGTGCTTTTGATGTCTGGGGTCAAGGGACG

e8 LC

ATGGCCGAGCTCACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGG

GGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCACAGAGTCAATAACA

ACTTCTTAGCCTGGTATCAGCAGAAACCTGGCCAGGCTCCCAGGCTC

CTCATCTCTGGTGCATCTACCAGGGCCACTGGCATCCCAGACAGGTT

CAGTGGCAGTGGGTCTGGAACAGACTTCACTCTCACCATCAGCAGAC

TGGAGCCTGATGATTTTGCAGTTTATTATTGTCAGCAGTATGGTGACT

CACCTCTTTATTCTTTTGGCCAGGGGACC

e10 HC

CTGCTCGAGTCTGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGT

CCCTCACCTGCACCGTCTCCGGTGTCTCCATCAGTTACGGTGGTCGT

GGCGTTTCCTACTGGGGTTGGGTCCGCCAGTCCCCAGGGAAGGGCC

TGGAGTGGATTGGCCACATCTACTACTTTGGAGACACCTTCTACAAC

CCGTCCCTCAACAATCGAGCTACCATATCAATAGACTCATCCAAAAAC

CAGTTCTCCCTCAAGCTCAAGTCTGTGACTGCCTCAGACACGGCCCT

GTATTTCTGTGCCAGGAGCACCCTACAGTATTTTGACTGGTTATTGAC

ACGGGAGGCTGCCTACTCCATTGACTTCTGGGGCCAGGGAATA

e10 LC

ATGGCCGAGCTCACCCAGTCTCCATCCTTCCTGTCTGCATCTGTTGG

AGACCGAGTCACCATCACTTGCCGGGCCAGTCAGGGCGTCACCATT

CTTTTAGCCTGGTATCAGCAAAAGCCAGGGAAACCCCCTAAGGCCCT

GATTTATGCTGCATCGTCTTTGCAAAGTGGGGTCCCATCAAGGTTCA

-continued

GCGGCAGTGGTTCTGACACAGATTTCACTCTCACAATCAGCAGCCTA

CAGCCTGAAGATTCTGCAACTTATTACTGTCAACAACTTAACACTTAC

CCGTGGACGTTCGGCCAGGGGACC

e20 HC

CTGCTCGAGCAGTCAGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGG

TGAAGGTCTCCTGCAAGGCTTCTGGAGACCACTATGGTATCAACTGG

GTGCGACAGGCCCCTGGACAAGGGCTGGAGTGGATGGGCGGTATCA

TCCCTGTCTTTGGCACAACTACCTACGCACAGAAGTTCCAGGGCAGA

GCCACCATTACCGCGGACGACTCCACGGGGACGGCCTTTTTGGAGC

TGACCAGACTGACATTTGACGACACGGCCGTCTATTTCTGTGCGACA

CCTCACCAACTGCATGTCCTCCGGGGCGGTAAAGCCCTCTCCCCCT

GGGACTACTGGGGCCAGGGAACC

e20 LC

ATGGCCGAGCTCACCCAGTCTCCAGCCACCCTGTCTGTGTCTCCAGG

GGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGT

AACTTAGCCTGGTACCAGCAGAAACGTGGCCAGGCTCCCAGTCTCCT

CATCTACGGAACATCTACCAGGGCCACTGGTATCCCAGCCAGGTTCA

GTGGCAGTGGGTCTGGGACAGAGTTCACTCTCACCATCAGCAGCCT

GCAGTCTGAAGATTTTGCAGTTTATTACTGTCAGCAGTATAATGATTG

GCCCTCCACCTTCGGCCAAGGGACA

e137 HC

CTGCTCGAGCAGTCTGGGTCTGAAGTAAAAGTGCCCGGGTCCTCGTT

GAAGGTCTCCTGCAAGACTTCTGGAGGCACCTTCAGCACCTATACTT

TCAGCTGGGTGCGACAGGCCCCTGGACAGGGACTTGAGTGGATGGG

GGGGATCACCCCTATCATTGGCATCGCAAACTACGCACGGAACTTCC

AGGACAGAGTCACCATCACCGCGGACGAATCCACGAGCACGGTCTA

CATGGAGGTGAGGAGGCTGAGATCTGAGGACACGGCCGTATATTATT

GTGCGAAAACTTCGGAAGTAACAGCCACTAGAGGGCGGACTTTCTTC

TACTCCGCTATGGACGTCTGGGGTCAAGGGACC

e137 LC

ATGGCCGAGCTCACCCAGTCTCCATCCTTCCTGTCTGCATCTGTAGG

AGACAGAGTCACCATCACTTGCCGGGCCAGTCAGGGCATAAGCAATT

ATTTAGCCTGGTATCAGCAAAAACCAGGGAAAGCCCCTAAGCTCCTG

ATCTATGCTGCATCCACTTTGCAAAGTGGGGTCCCATCGAGGTTCAG

CGGCAGTGGATCTTGGACAGAATTCACTCTCACAATCAGCCGCCTCC

AGCCTGAAGATTTTGCAACTTATTACTGTCAACACCTTAATACTTACCC

GTGGACGTTCGGCCAAGGGACC

-continued

e301 HC

CTGCTCGAGCAGTCTGGGTCTGAGGTGAAGAAACCTGGGTCCTCGG

TGAGGGTCTCGTGCACGACTTCTGGAGGCACCTTGAGCGACTATGGT

TTCAACTGGTTACGACAGGCCCCTGGACAAGGGCCTGAGTGGATGG

GAGGGATCATCCCTTTGTTTCGAAGAACAACCTACGGACAGAAGTTC

CAGGGCAGACTCACCATTACCGCGGACGAGTCCACGGGCGCAACCT

ACATGGAGCTGAGCAGCCTGAGATCTGACGACACGGCCGTCTATTAC

TGTGCGAGAGAGAAAGTTTCGGTCCTCACAGGCGGAAAGTCACTCCA

TTACTTTGAATATTGGGGCAAGGGAACC

e301 LC

ATGGCCGAGCTCACGCAGTCTCCAGCCACCCTGTCTGTGTCTCCAG

GGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAG

CAGGTTAGCCTGGTACCAGCAGAAACGTGGCCAGGCTCCCAGTCTC

CTCATCTATGACACATCTTCCAGGGCCACTGGTGTCCCAGCCAGGTT

CAGTGCCAGTGGGTCTGGGACGCAGTTCACTCTCACCATCAGCAGC

CTGCAGTCTGAAGATTTTGCACTTTATTACTGTCAGCAGTATAATGATT

GGCCCTCCACCTTCGGCCAAGGGACA

e509 HC

CTGCTCGAGGAGTCTGGGGCTGAGGTGAAGAAGCCAGGGTCCTCGG

TGAAGGTCTCCTGCAAGACTTCTGGAGACACCTTCAGATATGGTATC

ACGTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAC

AGATCATGCCTACGTTTGCGACAGCAACCTACGCACAGAGGTTCCAG

GGCAGAGTCACGATTTCCGCGGACGAATCCACGAGCACAGCCTACTT

GGAGGTGCGCAGCCTGAGATCTGAAGACACGGCCGTCTATTACTGT

GCGACACCTCGCCAAGTTACTATACTTCGGGGACCTAAAGCCCTCTC

CCCTTGGGACTACTGGGGCCAGGGAACC

e509 LC

ATGGCCGAGCTCACCCAGTCTCCAGCCACCCTGTCTGCGTCTCCAG

GGGAAAGAGCCTCCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGTAG

CAACTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTC

CTCATCTCTGGTGCATCCACCAGGGCCACTGGTGTCCCGGCCAGGT

TCAGTGGCAGTGGGTCTGGGACAGAGTTCACTCTCACCATCAGTAGC

CTGCAGTCTGAAGATTTTGCAGTTTATTACTGTCAGCAGTATAATAAC

TGGCCTCCCCACTTTGGCCAGGGGACC

[0036] FLAG-Fab ELISA on purified labeled Fab molecules yields very specific and reproducible results. Determination of FIT is performed on 10 HCV-negative sera; the titer is consistently >1:20, the upper detection limit of our test, indicating that no inhibition occurs in the absence of specific anti-HCV antibodies.

[0037] To demonstrate that FIT effectively measures the antibodies directed against epitopes recognized by our FLAG-Fabs, the same analysis is performed on mock specimens prepared by mixing negative sera with human monoclonal antibodies of given specificity, obtaining false samples containing known amounts of IgG directed against the HCV E2 epitopes defined by our Fabs. Results (**FIGS. 2A** and B) show a good correlation between FIT and antibody amount, indicating that FIT can provide reliable information on the amount of epitope-specific antibodies in a patient's serum.

[0038] Finally, FIT is always positive in HCV-positive sera, with values encompassing a wide range of dilutions. FIT is very diverse for the different Fabs in the same serum sample, with considerable heterogeneity between patients.

EXAMPLE 2

[0039] Materials and Methods

[0040] Human Antibody Fragments

[0041] The human recombinant antibody fragments in this example are fully described in Bugli et al. (2001) [71] and correspond to those used in Example 1. Briefly, genes coding for the Fabs were obtained from a phage display combinatorial library containing the IgG1/kappa repertoire of a 58-year-old woman with chronic hepatitis with persistent presence in the blood of HCV RNA of genotype 1b. The genes selected are inserted in an appropriate bacterial expression vector [13] and the transformed cells are then used as a source of recombinant Fabs, which are produced and purified as described [14]. Neutralization of E2 binding to cell (NOB) activity [5, 15] and the reciprocal interactions [7] of these molecules have been described. The presence of similar antibodies in the serum of HCV-infected patients is determined by inhibition ELISA [7].

[0042] Pseudotypes and Neutralization Assay

[0043] The pseudotypes used here have been fully characterized and described in Matsuura et al., 2001 [8]. Briefly, the VSVΔG*/HCVE1-E2 pseudotype (VSV/HCV) consists of Vesicular Stomatitis Virus, where the G envelope protein is replaced with chimeric E1 and E2 HCV envelope glycoproteins consisting of the ectodomains of E1 and E2 proteins of type 1 b HCV cDNA clone (NIH-J1) fused to the N-terminal signal sequences, with transmembrane and cytoplasmatic domains of VSV G protein [8]. The construction of plasmids [16], and eukaryotic expression vectors has been described [8, 17]. VSV/HCV is prepared by infecting CHO cells constitutively expressing chimeric E1 and E2 cDNA with a recombinant VSV in which the G protein-coding region has been replaced with the green fluorescent protein gene (GFP) [18]. The VSVΔG*/HCVE1-E2 (VSV/G) pseudotype used as control (and to produce the VSV/HCV pseudotype), is produced by infecting with VSVΔG* a cell line transiently expressing G protein. The neutralization assay is performed as described [8]. Dilutions of purified human recombinant Fabs are incubated with 2.4×10^3 Infection Units (IU) of the pseudotype VSV/HCV or VSV/G for 30 min at 37° C. and inoculated into HepG2 cells ($4\times^4$ cells) prepared in a 96-well plate. After adsorption for 60 min at 37° C., the cells are washed 3 times with DMEM containing 10% FBS and incubated at 37° C. for 16 hr. The IU of the virus are determined by counting the number of GFP-

expressing cells by fluorescence microscopy. Data are presented as percent of inhibition compared with control wells where no antibody was added. Data are the average of three experiments performed in double.

[0044] Results

[0045] Anti-HCV/E2 Human Monoclonal Antibody Panel Generation and Sequence Characterization

[0046] The panel of human monoclonal antibody Fab fragments represents the anti-HCV/E2 immune repertoire of a patient with a persistent infection with HCV of genotype 1b [5, 19]. Antibody fragments, selected with purified recombinant HCV/E2 of 1a genotype (strain H)[20] expressed in CHO cells, have been fully characterized and correspond to clones present in the serum of chronically infected patients [7] with a shared equal affinity for HCV/E2. Each of the five antibodies represents one of the five families in which the whole anti-E2 antibody repertoire of this patient is grouped. Fabs belonging to the same family share similar biological activity and have strong homologies of DNA sequences [5]. Each of the five Fabs recognizes a different epitope on the surface of E2 [7]. Divergences from the relative germ-line sequences are typical of antigen-driven affinity maturation (Tables 1a and 1b), suggesting a prolonged exposure to the antigen.

[0047] TABLES 1A, B. Germlines and V gene mutations in variable regions of anti-HCVE2 human monoclonal antibodies.

[0048] Sequences are determined as described in Burioni et al., 1998 [5] and aligned with germine sequences in the IMGT database [21]. The percentage of nucleotide and amino acid mutations are calculated according to the Kabat and Wu alignment method [22], taking into account framework region (FR) 1, FR 2 and FR 3 for heavy and light chains, the complementarity determining region (CDR) 1 and CDR 2 for heavy chains, CDR 1, CDR 2 and CDR 3 for light chains.

TABLE 1a

		HEAVY CHAINS			
		% of mutated nucleotides		% of mutated amino acids	
Antibody	V gene	FRs	CDRs	FRs	CDRs
e 8	VH1-18	9.5	22.2	14.9	33.3
e 20	VH1-69	9.4	16.9	19	38
e 137	VH1-69	11.5	15.3	14	41.7
e 301	VH1-69	8.9	19.4	15.6	45.8
e 509	VH1-69	5.2	15.9	10.9	33.3

[0049]

TABLE 1b

		LIGHT CHAINS			
		% of mutated nucleotides		% of mutated amino acids	
Antibody	V gene	FRs	CDRs	FRs	CDRs
e8	KV 3-20	2.7	16	2.6	33.3
e20	KV 1-9	4.3	7.7	9.7	22.2
e137	KV 1-8	2.2	9	3.2	15.4

TABLE 1b-continued

		LIGHT CHAINS			
		% of mutated nucleotides		% of mutated amino acids	
Antibody	V gene	FRs	CDRs	FRs	CDRs
e301	KV 3-15	3.8	14.3	9.7	23
e509	KV 3-15	3.2	1.3	6.5	0

[0050] Neutralizing of binding (NOB) activity of each Fab was also determined [5], with some clones (e137 and e8) found to be unable to inhibit HCV/E2 binding to cells and others inhibiting HCV/E2 binding even at very low concentration (see below).

[0051] Neutralization of the Pseudotype Virus by Human Recombinant Fabs

[0052] Two of the Fabs, e8 and e20, recognizing different epitopes on the surface of HCV/E2 [7] do not neutralize VSV/HCV pseudotype infection even at high concentrations (80 μg/ml). One of these two Fabs, e20, has strong NOB activity [5], confirming that even antibodies inhibiting E2 binding may fail to prevent viral infection.

[0053] Two other Fabs, e137 and e301, efficiently neutralize VSV/HCV at a concentration of 10 μg/ml, while VSV pseudotypes bearing the VSV G envelope protein (VSV/G pseudotypes) are not affected (**FIGS. 3*a*** and **3*b***). These data are congruent with previous findings indicating that these two clones compete for the same E2 region, probably recognized by human antibodies endowed with neutralizing activity, as indicated in a two-dimensional surface map of the human epitopes on HCV/E2 (**FIG. 4**). Fab 509 is currently the strongest available antibody in terms of NOB activity, and is able to inhibit binding between E2 and the cellular target at very low concentrations (Table 2). Incubation of VSV/HCV pseudotypes with this Fab enhance virus entry into hepatoma cells down to a concentration of 1 μg/ml. No increase in infectivity is demonstrated when VSV/G pseudotypes are used, thus ruling out the possibility that a non-specific interaction of this Fab with cellular membrane promotes viral entry into the cell (**FIG. 3C**).

TABLE 2

Anti-HCV/E2 antibodies characteristics
NOB activity is calculated as the concentration (in μg/ml) achieving 50% of neutralization of binding of a purified HCV/E2 preparation to cellular targets.

Fab clone	50% NOB concentration (μg/ml)	Effect on VSV/HCV infection
e8	>40 (none)	none
e20	3 (high)	none
e137	40 (low)	inhibition
e301	3 (high)	strong inhibition
e509	<0.035 (highest)	enhancement

[0054] A control antibody [23] exerts no effect on the pseudotype system, as it fails to neutralize both VSV/HCV and VSV/G pseudotypes. The VSV/G pseudotype is duly neutralized by dilutions up to 1:1000 of a polyclonal anti-VSV antiserum used as neutralizing control in these experi-

ments [8], which have no effect on the VSV/HCV. Polyclonal and monoclonal anti-E1 and anti-E2 antibodies raised in several hosts show no neutralizing effect on VSV/HCV pseudotypes.

[0055] The neutralizing activity of monovalent Fabs shows that HCV entry can be inhibited without the need for virion aggregation or cross-linking; furthermore, blocking of interaction between the virus and its cellular target seems unlikely to be a key factor in HCV neutralization. These data can explain at the molecular level the lack of correlation between NOB activity in the serum and protection from disease.

[0056] Some degree of cross-protection is provided by anti-HCV antibodies, as anti-E2 antibodies selected with E2 of 1a genotype are able to neutralize a pseudotype bearing E2 of 1b genotype.

[0057] The results show that Fab 509 is able to enhance the infectivity of the VSV/HCV pseudotype virus, although no effect on the VSV/G construct was apparent. A possible explanation for the ability of e509 to promote viral entry can be found in the observation that this antibody binds specifically and very efficiently to the region of E2 that binds to CD81, a cellular structure involved in viral attachment to the cell [24]. The binding of e509 to E2 could mimic the binding of E2 to one of its cellular targets and promote a modification of E2 conformation similar to the one induced by CD81. E2 is present in at least two conformational states and antibody binding to this protein can modify the sterical status of the protein by modulating the NOB activity of human Fabs without binding competition [6]. Hence, Fab 509 seems to be a key tool for the study of the interactions between HCV and the cell surface and could be used in in vitro models for the evaluation of molecules for vaccines.

[0058] References

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ctgacatttg acgacacggc cgtctatttc tgtgcgacac ctcaccaact gcatgtcctc 300

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ggccaggctc ccagtctcct catctacgga acatctacca gggccactgg tatcccagcc 180

aggttcagtg gcagtgggtc tgggacagag ttcactctca ccatcagcag cctgcagtct 240

gaagattttg cagtttatta ctgtcagcag tataatgatt ggccctccac cttcggccaa 300

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<211> LENGTH: 360
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<400> SEQUENCE: 19

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cagggacttg agtggatggg ggggatcacc cctatcattg gcatcgcaaa ctacgcacgg 180

aacttccagg acagagtcac catcaccgcg gacgaatcca cgagcacggt ctacatggag 240

gtgaggaggc tgagatctga ggacacggcc gtatattatt gtgcgaaaac ttcggaagta 300

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<400> SEQUENCE: 20

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gggaaagccc ctaagctcct gatctatgct gcatccactt tgcaaagtgg ggtcccatcg 180

aggttcagcg gcagtggatc ttggacagaa ttcactctca caatcagccg cctccagcct 240

gaagattttg caacttatta ctgtcaacac cttaatactt acccgtggac gttcggccaa 300

gggacc 306

<210> SEQ ID NO 21
<211> LENGTH: 354
<212> TYPE: DNA
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caagggcctg agtggatggg agggatcatc cctttgtttc gaagaacaac ctacggacag 180

aagttccagg gcagactcac cattaccgcg gacgagtcca cgggcgcaac ctacatggag 240

ctgagcagcc tgagatctga cgacacggcc gtctattact gtgcgagaga gaaagtttcg 300

gtcctcacag gcggaaagtc actccattac tttgaatatt ggggcaaggg aacc 354

<210> SEQ ID NO 22
<211> LENGTH: 306
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

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ggccaggctc ccagtctcct catctatgac acatcttcca gggccactgg tgtcccagcc 180

aggttcagtg ccagtgggtc tgggacgcag ttcactctca ccatcagcag cctgcagtct 240

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<210> SEQ ID NO 23
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gggcttgagt ggatgggaca gatcatgcct acgtttgcga cagcaaccta cgcacagagg 180

ttccagggca gagtcacgat ttccgcggac gaatccacga gcacagccta cttggaggtg 240

cgcagcctga gatctgaaga cacggccgtc tattactgtg cgacacctcg ccaagttact 300

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ggccaggctc ccaggctcct catctctggt gcatccacca gggccactgg tgtcccggcc 180

aggttcagtg gcagtgggtc tgggacagag ttcactctca ccatcagtag cctgcagtct 240

gaagattttg cagtttatta ctgtcagcag tataataact ggcctcccca ctttggccag 300

gggacc 306

1. An human antibody, or its functional fragments, against the HCV E2 protein characterized in having an in vivo neutralizing activity.

2. Antibody according to claim 1, being the antibody e137 characterized by having the following sequences of variable parts of the heavy chain and the light chain:

e 137 Heavy chain (HC)

LLEQSGSEVKVPGSSLKVSCKTSGGTFSTYTFSWVRQAPGQGLEWMGGITPIIGIA

NYARNFQDRVTITADESTSTVYMEVRRLRSEDTAVYYCAKTSEVTATRGRTFFYSA

MDVWGQGT

e 137 Light chain (LC)

MAELTQSPSFLSASVGDRVTITCRASQGISNYLAWYQQKPGKAPKLLIYAASTLQS

GVPSRFSGSGSWTEFTLTISRLQPEDFATYYCQHLNTYPWTFGQGT

3. Antibody according to claim 1 being the antibody e301 characterized by having the following sequences of variable parts of the heavy chain and the light chain:

e 301 Heavy chain (HC)

LLEQSGSEVKKPGSSVRVSCTTSGGTLSDYGFNWLRQAPGQGPEWMGGIIPLFRRT

TYGQKFQGRLTITADESTGATYMELSSLRSDDTAVYYCAREKVSVLTGGKSLHYFE

YWGKGT

e 301 Light chain (LC)

MAELTQSPATLSVSPGERATLSCRASQSVSSRLAWYQQKRGQAPSLLIYDTSSRAT

GVPARFSASGSGTQFTLTISSLQSEDFALYYCQQYNDWPSTFGQGT

4. (canceled)

5. (canceled)

6. (canceled)

7. (canceled)

8. (canceled)

9. (canceled)

10. (canceled)

11. (canceled)

12. A method for validating anti-HCV vaccines using the antibody according to claim 1.

13. A method for validating anti-HCV vaccines using the antibody according to claim 2.

14. A method for validating anti-HCV vaccines using the antibody according to claim 3.

15. A nucleic acid coding for the antibody according to claim 1.

16. A nucleic acid coding for the antibody according to claim 2.

17. A nucleic acid coding for the antibody according to claim 3.

18. A recombinant expression vector expressing the antibody of claim 1 in prokaryote or in eukaryote cells.

19. A recombinant expression vector expressing the antibody of claim 2 in prokaryote or in eukaryote cells.

20. A recombinant expression vector expressing the antibody of claim 3 in prokaryote or in eukaryote cells.

21. A recombinant vector further comprising a nucleotide sequence coding for a signal peptide, substantially contiguous with the sequence coding for the antibody of claim 1, able to export this antibody out of the cell environment.

22. A recombinant vector further comprising a nucleotide sequence coding for a signal peptide, substantially contiguous with the sequence coding for the antibody of claim 2, able to export this antibody out of the cell environment.

23. A recombinant vector further comprising a nucleotide sequence coding for a signal peptide, substantially contiguous with the sequence coding for the antibody of claim 3, able to export this antibody out of the cell environment.

24. A method of using the recombinant vector according to claim 21 in gene therapy.

25. A method of using the recombinant vector according to claim 22 in gene therapy.

26. A method of using the recombinant vector according to claim 23 in gene therapy.

27. A composition for anti-HCV therapy comprising in a therapeutically effective amount at least one of the antibodies according to the claims.

28. A composition according to claim 27 for topical use in gel, creme, ointment and ovule formulations.

29. A method for the determination of the presence of antibodies directed against different epitopes of the HCV E2 protein in a biological fluid comprising the steps of:

a) determining the presence of antibodies in said fluid able to inhibit the binding of specific human Fab directed against different epitopes of protein E2; and

b) correlating the presence of so titered antibodies with clinical characteristics of patients, such as prognosis, responsiveness to therapy, infectivity.

* * * * *