EPITOPES IN VIRAL ENVELOPE PROTEINS AND SPECIFIC ANTIBODIES DIRECTED AGAINST THESE EPITOPES: USE FOR DETECTION OF HCV VIRAL ANTIGEN IN HOST TISSUE

FIELD OF THE INVENTION

The present invention is based on the finding that antibodies directed against specific epitopes of the E1 and E2 protein of HCV can be used to detect viral antigens in host tissues.

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) infection is a major health problem in both developed and developing countries. It is estimated that about 1 to 5 % of the world population is affected by the virus. HCV infection appears to be the most important cause of transfusion-associated hepatitis and frequently progresses to chronic liver damage. Moreover, there is evidence implicating HCV in induction of hepatocellular carcinoma. Consequently, the demand for reliable diagnostic methods and effective therapeutic agents is high. Also sensitive and specific screening methods of HCV-contaminated blood-products and improved methods to culture HCV are needed.

HCV is a positive stranded RNA virus of approximately 9,400 bases which encode at least three structural and six non-structural proteins. Based on sequence homology, the structural proteins have been functionally assigned as one single core protein and two envelope proteins: E1 and E2. The E1 protein consists of 192 amino acids and contains 5 to 6 N-glycosylation sites, depending on the HCV genotype. The E2 protein consists of 363 to 370 amino acids containing 9 to 11 N-glycosylation sites, depending on the HCV genotype (for review see Major and Feinstone, 1997; Maertens and Stuyver, 1997). The E1 protein contains various variable domains, while the E2 protein contains two hypervariable domains, of which the major domain is located at the N-terminus of the protein (Maertens and Stuyver, 1997). The envelope proteins have been produced by recombinant techniques in *Escherichia coli*, insect cells, yeast cells and mammalian cells. The usage of an expression system in higher eukaryotes and especially in mammalian cell culture leads to envelope proteins of superior quality, i.e. they are effectively recognised by antibodies in patient samples as described in PCT/EP 95/03031 to Maertens et al.

Currently, the detection of HCV in cells or tissues relies mainly on the demonstration of viral RNA. However, RNA detection in cells or tissues is a cumbersome technique which either involves the extraction of RNA followed by reverse transcription and nested PCR or includes *in situ* RT-PCR and hybridisation. Since these techniques are also prone to false positive reactivity, viral RNA detection is exclusively performed on serum samples. Reliable methods for the detection of viral protein antigens in serum and tissue samples are still lacking.

The replication sites of HCV have not yet been fully elucidated. It is generally accepted that the virus replicates in hepatocytes, but replication in other tissues, such as lymphoid tissues, is still highly debated. Hence, a reliable method for the detection of viral proteins or the virus itself in cells may solve this issue.

The detection of viral proteins in cells has been hampered by the lack of antibodies which specifically bind to viral proteins and which are able to specifically recognise the natural HCV protein antigens (i.e. antigens as expressed by the host following infection with HCV). As a consequence, to date only few studies relate to demonstrating the presence of viral HCV proteins in host cells (for review see Guido and Thung, 1996). Moreover, host-derived antibodies have been used in many of these studies. Preparations containing host-derived antibodies, however, cannot be reproduced easily and these preparations may be contaminated by autoimmune antibodies as well as by antibodies against other known or even unknown agents. In contrast, it is known that antibodies produced in animals upon immunisation with recombinant antigens will yield antibodies with the desired specificity. In order to have reproducible quality, monoclonal antibodies are preferred as well. In addition, the envelope proteins of HCV need to be produced by a mammalian expression system to yield good quality antigens. Currently, this expression condition goes together with incomprehensible problems. Therefore, only few monoclonal antibodies have been described which could be used to detect HCV envelope antigens in tissue specimens of patients. These antibodies were directed against the N-terminal region of E1, amino acids (aa) 192-226, (Hiramatsu et al., 1992, Kaito et al., 1994) or the C-terminal domain of E2, aa 451-715 (Sansonno et al., 1997a, 1997b). However, from these publications and from the reviews by Guido and Thung (1996), and Liang (1996) it is evident that there is still an existing need for well-characterised antibodies allowing efficient and routine detection of natural HCV protein antigens in serum and tissue samples. This need was recently confirmed by a study by Dries and co-workers (1999). Dries' group proved that 61% of HCV antibody positive and serum RNA negative individuals were carriers of HCV as HCV RNA could be detected in liver biopsy

samples but only one third of these cases could be detected by immunohistochemistry using a panel of antibodies. Thus, routine detection of HCV antigen in liver biopsies still lacks sensitivity. Also the detection of viral antigens in body fluids such as serum or plasma is hampered by the same problem. Up to date only one single technique has been described allowing detection of core antigen in these fluids. However, in order to detect the core protein, the technique requires a complete denaturation of the core protein present in the sample using sodium hydroxide (Kashiwakuma et al., 1996).

Taken together, the identification of new specific HCV epitopes which are accessible for antibodies and which allow antigen detection in tissue or body fluid samples is therefore urgently needed. The HCV envelope proteins are putative candidate targets to find such epitopes, since these proteins should be present in all biological samples: in fluids, on the membrane of the virus, and in cells from the earliest event of infection (i.e. viral entry) throughout the complete replication cycle. However, these envelope proteins are highly variable so antibodies with a high cross-reactivity towards the different genotypes of HCV are needed. Thus, identification of such epitopes and the search for antibodies with high cross-reactivity towards the sequence variation of HCV is a challenging undertaking.

The present application relates to specific monoclonal antibodies, directed against particular epitopes in the envelope proteins of HCV, which are able to detect HCV antigen in tissue specimens of patients. In total two such epitopes, and corresponding antibodies, were found: one in the C-terminal region (aa 227-383) of the envelope protein E1 and one in the N-terminal hypervariable region (HVR) of E2 (aa 384-450). Although the latter region, and more specifically the region 395-415, is considered to be hypervariable, we characterised, to our surprise, an antibody which reacts with various known sequences of the HVR of E2.

AIMS OF THE INVENTION

It is clear from the literature that there is an urgent need to develop reliable diagnostic methods, reliable vaccines and effective therapeutic agents for HCV. Also sensitive and specific screening methods of HCV-contaminated blood-products and improved methods to culture HCV are needed. New antibodies able to detect the virus in animal- or *in vitro* models, or in its natural host, may help in designing efficient diagnostic tools and therapeutic agents. In this regard, the

present invention is based on the surprising finding of monoclonal antibodies directed against either E1 or E2-HVR which can be used for the detection of HCV antigens in various tissues or cells. These tissues include the liver but also cells derived from blood samples. Therefore, the present invention aims at providing and using an antibody specifically binding to HCV envelope protein region aa 227-450, which covers the main part (C-terminal) of the E1 protein, and the N-terminal region of the E2 protein. These antibodies allow detection of natural HCV protein antigens, and can be used for the preparation of a natural HCV protein antigen detection kit. Notably, the complete E1 protein corresponds to aa 192-383, while the complete E2 protein corresponds to aa 384-747 (see: Major and Feinstone, 1997; Maertens and Stuyver, 1997).

More specifically, the present invention aims at providing and using an antibody as defined above which specifically binds to at least one of the following epitopes:

-aa 307-326 of HCV E1 protein (SEQ ID 30)

-aa 395-415 of HCV E2 protein (SEQ ID 31).

Moreover, the present invention aims at providing and using an antibody as defined above which is a monoclonal antibody. In this regard, the present invention aims at providing and using a monoclonal antibody secreted by the hybridoma line with ECACC deposit having accession number 98031215 or 98031214.

It should be clear that the present invention also aims at providing and using any functionally equivalent variant or fragment of any antibody as defined above, as well as mutant forms thereof, or molecules exhibiting similar functional binding reactivities with SEQ ID 30 and 31, such as sequences obtained from phage- or other libraries.

In addition, the present invention aims at providing and using a hybridoma cell line secreting a monoclonal antibody which specifically binds to HCV E1 protein (aa 227-383) or HCV E2 N-terminal hypervariable region (aa 384-450) and which allows the detection of natural HCV protein antigens, and can be used for the preparation of a natural HCV protein antigen detection kit. More specifically, the present invention aims at providing and using the hybridoma cell line corresponding to the ECACC deposit having accession number 98031215 or 98031214.

Furthermore, the present invention also aims at providing a method for the detection of natural HCV protein antigens comprising:

- contacting a test sample which may contain HCV protein antigens with an antibody as defined above or with a functionally equivalent variant or fragment of said antibody, to form an antibody-antigen complex, and

- determining said antigen-antibody complex with an appropriate marker.

More specifically, the present invention aims at providing a method as defined above wherein said test sample comprises human cells, such as peripheral blood cells, or human tissues, such as liver tissue.

Finally, the present invention aims at providing an assay kit for the detection of natural HCV protein antigens comprising:

- an antibody as defined above, or, a functionally equivalent variant or fragment of said antibody, and
- appropriate markers which allow to determine the complexes formed between HCV protein antigens in a test sample with said antibody or a functionally equivalent variant or fragment of said antibody.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

BRIEF DESCRIPTION OF TABLES AND DRAWINGS

Table 1 provides the peptide sequences of all peptides mentioned in this application.

Table 2 shows the cross-reactivity of IGH 222 towards various sequences of the hyper-variable domain in E2. All peptides were biotinylated, bound to streptavidin-coated microtiterplates and allowed to react with IGH 222.

Figure 1 shows staining of E2 antigen, revealed by the monoclonal antibody IGH 222, on a liver biopsy of an HCV patient. Immunohistochemistry was performed on 4 µm thick cryostat sections of fresh frozen materials (the liver biopsy was snap-frozen in liquid nitrogen-cooled isopentane and stored at -70°C until use) using a three step indirect immuno-peroxidase procedure. Sections were incubated overnight at 4°C with monoclonal antibody IGH 222 (purified IgG_1: 10ng/µl). The secondary and tertiary antibodies consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit IgG, respectively (both obtained from Dakopatts, Copenhagen, Denmark; working dilution 1/50 and 1/100, respectively). Each incubation was performed 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2. The reaction product was developed by incubation for 15 minutes in

100mM acetate buffer (pH 5.2), containing 0.05% 3-amino-9-ethyl-carbazole and 0.01% H_2O_2, resulting in bright red staining of immuno-reactive sites. The sections were counterstained with haemotoxylin. Controls (not shown) consisted of irrelevant monoclonal antibodies of similar isotype as the primary antibody, or of chromogen alone: these controls were consistently negative. The photograph shows a 25X magnification. The darkest staining reveals the presence of HCV antigen in hepatocytes only (see arrows).

Figure 2A shows staining of E1 antigen, revealed by the monoclonal antibody IGH 207, on a liver biopsy of an HCV patient. The procedure followed is identical to the one described in figure 1, except for the concentration of the monoclonal antibody which was 30 ng/µl. The photograph shows a 10X magnification on which the staining of the cells in the lymphocyte infiltrates is dominant. The darkest staining reveals the presence of HCV antigen in hepatocytes (see arrow) and in infiltrating lymphocytes (see double arrow).

Figure 2B shows staining of E1 antigen, revealed by the monoclonal antibody IGH 210, on a liver biopsy of an HCV patient. The liver biopsy was fixed with formaldehyde and embedded in paraffin. Sections were pretreated by heat (microwave method) and by protease digestion. The concentration of the antibody was 6ng/µl. The photograph shows a clearly stained isolated mononuclear cell (arrow) in a field of hepatocytes, which do not stain with this monoclonal.

Figure 3 shows staining, revealed by the monoclonal antibody IGH 207, of intracellular E1 antigen in peripheral blood mononuclear cells. Peripheral white blood cells ($0.5x10^6$) were suspended in 200 µl PBS-0.1% saponin, 2 µg of IGH 207 was added and allowed to react for 25 minutes at 4°C. The cells are washed three times with 3 ml PBS-0.1% saponin and three times with PBS-0.2% NaN_3. Finally cells are resuspended in 250 µl of PBS-0.2% NaN_3 and analysed by flow cytometry. After gating on the mononuclear cell fraction (right column), the fluorescence was plotted (left column). Samples 1-5 are derived from HCV chronic carriers while sample 6 is derived from a healthy blood carrier. The left column shows two examples of the gating on the mononuclear cell fraction (samples 2 and 6), while the right column shows the fluorescence found in these mononuclear cells. While the control sample shows no staining at all with this monoclonal antibody, there is a marked positive signal in all HCV patients, except for patient 4 for whom a weaker signal was obtained.

Figure 4 shows staining, revealed by the monoclonal antibody IGH201, of intracellular E1 antigen in peripheral blood mononuclear cells. The technique was similar as described for Figure 3. Samples 7-11 are derived from HCV chronic carriers while sample 12 is derived from a healthy blood carrier. The left column shows two examples of the gating on the mononuclear cell fraction (samples 7 and 12), while the right column shows the fluorescence found in these mononuclear cells. Although the control sample reveals a higher background staining, the reaction in the patient samples can be easily discriminated based on the two populations which can be detected: a population with a similar staining as in the control and a second population with high intensity staining, not seen in the control.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

It is clear that the detection of viral proteins has been hampered by the lack of antibodies which specifically bind to viral proteins and which are able to recognise the natural HCV protein antigens (i.e. protein antigens as expressed by the host following infection with HCV). The present invention is based on the finding of two new epitopes on the HCV envelope proteins which allow routine detection of natural HCV protein antigens, by means of antibodies directed against these epitopes, in biological samples derived from the host. Thus, the present invention relates to the use of antibodies specifically binding to the C-terminal region of HCV E1 protein (aa 227-383) or the N-terminal region of the HCV E2 protein (aa 384-450) for the preparation of a natural HCV protein antigen detection kit. The term "antibodies specifically binding to the C-terminal region of the HCV E1 protein (aa 227-383) or the N-terminal region of the HCV E2 protein (aa 384-450)" refers to any polyclonal or monoclonal antibody binding to an hepatitis C viral particle or any molecule derived from said viral particle, more particularly to the C-terminal region of the E1 protein and the N-terminal region of the E2 protein. The "envelope region" of the HCV viruses, and thus "the C-terminal region of the HCV E1 protein (aa 227-383) or the N-

terminal region of the HCV E2 protein (aa 384-450)" are well-known regions by a person skilled in the art (Wengler, 1991; Major and Feinstone, 1997; Maertens and Stuyver, 1997).

The term "bind" indicates that the antibodies of the present invention are physically connected to HCV proteins. In particular, that the antibodies specifically bind to the HCV envelope proteins, implying that there is substantially no cross-reaction with other components of HCV or other proteins. Binding of the antibody to HCV proteins can be demonstrated by any method or assay known in the art, such as binding-, ELISA-, and RIA-type of assays or competition assays (e.g. see Current protocols in immunology). It should be clear that the region of an HCV envelope protein which binds to an antibody need not to be composed of a contiguous sequence.

The term "monoclonal antibody" used herein refers to an antibody composition having a homogeneous antibody population. The term is not limiting regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. In addition, the term "antibody" also refers to humanised antibodies in which at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences and single chain antibodies as described in U.S. patent N° 4,946,778 and to fragments of antibodies such as F_{ab}, $F_{(ab)2}$, F_v, and other fragments which retain the antigen binding function and specificity of the parent antibody. Also included in the term "antibody" are diabodies, triabodies and tetravalent antibodies, as described in EP application N° 97870092.0 to Lorré et al., which retain the antigen binding function and specificity of the parent antibody. It should be clear that the antibodies as described in the present invention could be used in a method for the detection of natural HCV protein antigens.

The term "test sample" refers to any sample obtained from a host, such as serum, plasma, saliva, mucus, spinal cord fluid, or biopsies. In this regard, the terms "test sample" and "biological sample" are used interchangeable herein. The term "biopsy" particularly refers to a sample comprising cells, in particular human cells. Moreover, the latter term refers also to a sample derived from liquid tissue, such as peripheral blood cells, or from solid tissue, such as liver tissue. In case antigen detection is performed on a biopsy, the term "*in situ* detection" is used.

The term "natural HCV protein antigen detection kit" refers to a kit for the detection of any HCV protein antigen, preferentially to the detection of the C-terminal region of the HCV E1 protein (aa 227-383) or the N-terminal region of the HCV E2 protein (aa 384-450) in their natural

or original position, preferentially the antigens as present in a test sample, by any method known to the man skilled in the art. In other words, the latter term refers to the visualisation of the presence of the C-terminal region of HCV E1 (aa 227-383) protein or the N-terminal region of the HCV E2 protein (aa 384-450) in, or on, their natural host cell or tissue by binding of the antibodies of the present invention. The detection kit comprises the following components:

(i) when appropriate a means for isolating a test sample,

(ii) possibly a solution to permeabilise cells,

(iii) an antibody as described herein, or a functionally equivalent variant or fragment thereof,

(iv) possibly secondary and possibly tertiary antibodies,

(v) possibly incubation and/or washing buffers,

(vi) possibly a staining solution.

Preferentially, said kit is used for the *in situ* detection of HCV envelope proteins.

The natural host cell or tissue can be any host cell or tissue derived from any host species. In particular, the natural host cell refers to peripheral blood cells and the natural tissue refers to liver tissue (see also *Examples* section of the present application). The natural host refers, in particular, to humans but may also refer to non-human primates or other mammals.

More specifically, the present invention relates to antibodies, which specifically bind to at least one of the following epitopes: aa 307-326 of HCV E1 protein and aa 395-415 of HCV E2 protein. The latter antibodies are secreted by hybridomas deposited at the European Collection of Cell Cultures (ECACC), Centre for Applied Microbiology & Research, Salisbury, Wiltshire SP4 OJG, U.K. (Tel: +44 1980 612512; fax: +44 1980 611315) on March 13, 1998, which have been assigned the following accession numbers: 98031215 for hybridoma 17H10F4D10 secreting mAb IGH 222, which binds epitope aa 395-415 (SEQ ID 31), and, 98031214 for hybridoma 14H11B2 secreting mAb IGH 207, which binds epitope aa 307-326 (SEQ ID 30). In this regard, it should be clear that antibodies that bind to parts of said epitopes are also part of the present invention. For example, antibodies binding to the following epitopes are hereby also included: aa307-311, aa308-312, aa309-313, aa310-314, aa311-315, aa312-316, aa312-317, aa313-318, aa314-319, aa315-320, aa316-321, aa317-322, aa318-323, aa319-324, aa320-325, aa321-326, aa307-312, aa308-313, aa309-314, aa310-315, aa311-316, aa312-317, aa313-318, aa314-319, aa315-320, aa316-321, aa317-322, aa318-323, aa319-324, aa320-325, aa321-326, aa 307-313, aa308-314, aa309-315, aa310-316, aa311-317, aa312-318, aa313-319, aa314-320, aa315-321, aa316-322, aa317-323, aa318-324, aa319-325, aa320-326, aa307-314, aa308-315, aa309-316,

aa310-317, aa311-318, aa312-319, aa313-320, aa314-321, aa315-322, aa316-323, aa317-324, aa318-325, aa319-326, aa307-315, aa308-316, aa309-317, aa310-318, aa311-319, aa312-320, aa313-321, aa314-322, aa315-323, aa316-324, aa317-325, aa318-326, aa307-316, aa308-317, aa309-318, aa310-319, aa311-320, aa312-321, aa313-322, aa314-323, aa315-324, aa316-325, aa317-326, aa307-317, aa308-318, aa309-319, aa310-320, aa311-321, aa312-322, aa313-323, aa314-324, aa315-325, aa316-326, aa307-318, aa308-319, aa309-320, aa310-321, aa311-322, aa312-323, aa313-324, aa314-325, aa315-326, aa307-319, aa308-320, aa309-321, aa310-322, aa311-323, aa312-324, aa313-325, aa314-326, aa307-320, aa308-321, aa309-322, aa310-323, aa311-324, aa312-325, aa313-326, aa307-321, aa308-322, aa309-323, aa310-324, aa311-325, aa312-326, aa307-322, aa308-323, aa309-324, aa310-325, aa311-326, aa307-323, aa308-324, aa309-325, aa310-326, aa307-324, aa308-325, aa309-326, aa307-325, aa308-326, and,

aa395-399, aa396-400, aa397-401, aa398-402, aa399-403, aa400-404, aa401-405, aa402-406, aa403-407, aa404-408, aa405-409, aa406-410, aa407-411, aa408-412, aa409-413, aa410-414, aa411-415, aa395-400, aa396-401, aa397-402, aa398-403, aa399-404, aa400-405, aa401-406, aa402-407, aa403-408, aa404-409, aa405-410, aa406-411, aa407-412, aa408-413, aa409-414, aa410-415, aa395-401, aa396-402, aa397-403, aa398-404, aa399-405, aa400-406, aa401-407, aa402-408, aa403-409, aa404-410, aa405-411, aa406-412, aa407-413, aa408-414, aa409-415, aa395-402, aa396-403, aa397-404, aa398-405, aa399-406, aa400-407, aa401-408, aa402-409, aa403-410, aa404-411, aa405-412, aa406-413, aa407-414, aa408-415, aa395-403, aa396-404, aa397-405, aa398-406, aa399-407, aa400-408, aa401-409, aa402-410, aa403-411, aa404-412, aa405-413, aa406-414, aa407-415,aa395-403, aa396-404, aa397-405, aa398-406, aa399-407, aa400-408, aa401-409, aa402-410, aa403-411, aa404-412, aa405-413, aa406-414, aa407-415, aa395-404, aa396-405, aa397-406, aa398-407, aa399-408, aa400-409, aa401-410, aa402-411, aa403-412, aa404-413, aa405-414, aa406-415, aa395-405, aa396-406, aa397-407, aa398-408, aa399-409, aa400-410, aa401-411, aa402-412, aa403-413, aa404-414, aa405-415, aa395-406, aa396-407, aa397-408, aa398-409, aa399-410, aa400-411, aa401-412, aa402-413, aa403-414, aa404-415, aa395-407, aa396-408, aa397-409, aa398-410, aa399-411, aa400-412, aa401-413, aa402-414, aa403-415, aa395-408, aa396-409, aa397-410, aa398-411, aa399-412, aa400-413, aa401-414, aa402-415, aa395-409, aa396-410, aa397-411, aa398-412, aa399-413, aa400-414, aa401-415, aa395-410, aa396-411, aa397-412, aa398-413, aa399-414, aa400-415, aa395-411, aa396-412, aa397-413, aa398-414, aa399-415, aa395-412, aa396-413, aa397-414, aa398-415, aa395-413, aa396-414, aa397-415, aa395-414 and 396-415,

The present invention also relates to functionally equivalent variants or fragments of the above-indicated antibodies. The term "functionally equivalent variants or fragments" refers to any variant or fragment known in the art of said antibodies which retain the antigen binding function and specificity of the parent antibody.

More specifically, the latter term refers to humanised antibodies and single chain antibodies as defined above and to fragments of antibodies such as F_{ab}, $F_{(ab)2}$, F_v, and the like. Also included are diabodies, triabodies and tetravalent antibodies, as described above, as well as mutant forms thereof or molecules exhibiting similar functional binding reactivities with SEQ ID 30 and 31, such as sequences obtained from phage- or other libraries as described by Ladner, 1995; Maclennan, 1995 and Cannon et al., 1996. Indeed, any peptide described by these authors, constrained or not, which allows the detection of natural HCV protein antigens is part of the present invention.

The present invention also relates to hybridoma cell lines secreting antibodies as defined above. In this regard, it should be clear that the hybridoma technology for obtaining mAbs is well known to a person skilled in the art, e.g. essentially according to Kohler and Milstein (1975). It should also be clear that mapping the epitopes to which the mAbs specifically bind can be performed by any method known in the art such as the ones described in PCT/EP 97/07268 to Depla et al.

The present invention also relates to a method for the detection of natural HCV protein antigens comprising:

- contacting a test sample which may contain HCV protein antigens with an antibody as defined above or with a functionally equivalent variant or fragment of said antibody, to form an antibody-antigen complex, and
- determining said antigen-antibody complex with an appropriate marker.

Preferentially, said method is used for the *in situ* detection of HCV envelope proteins.

The term "determining said antigen-antibody complex with an appropriate marker" refers to any method known in the art which detects, or visualises, the above-indicated antigen-antibody complexes, such as fluorescence flow cytometry, binding-, ELISA- and RIA-type assays or competition assays (see *Examples* section, Hertogs et al., 1993, and, WO 93/04084 to Mehta et al.). Similarly, the term "appropriate marker" refers to any marker known in the art such as the ones described in WO 93/04084 to Mehta et al. and Coligan et al., 1992 which visualises the

above-indicated antigen-antibody complexes.

In this regard, the present invention also relates to an assay kit for the detection of natural HCV protein antigens comprising: an antibody as defined above, or, a functionally equivalent variant or fragment of said antibody, and appropriate markers which allow to determine the complexes formed between HCV protein antigens in a test sample with said antibody or a functionally equivalent variant or fragment of said antibody.

The present invention will now be illustrated by reference to the following examples that set forth particularly advantageous embodiments. However, it should be noted that these embodiments are merely illustrative and can not be construed as to restrict the invention in any way.

EXAMPLES

Example 1: Generation of monoclonal antibodies against E1 and E2.

Mice were immunised with truncated versions of E1 (aa 192-326) and E2 (aa 384-673), expressed by recombinant vaccinia virus, as described in PCT/EP 95/03031 to Maertens et al. After immunisation splenocytes of the mice were fused with a myeloma cell line. Resulting hybridomas secreting specific antibodies for E1 or E2 were selected by means of ELISA.

Example 2: Selection of monoclonal antibodies.

A large series (25 in total, of which 14 in detail; the latter can be obtained from Innogenetics NV, Gent, Belgium) of monoclonals directed against E1 or E2 was evaluated for staining of native HCV antigen in liver biopsies of HCV patients and controls (see below). Staining was performed either on cryosections or on formaldehyde fixed biopsies (for protocols, see figure legends). Only three monoclonal antibodies revealed a clear and specific staining. All other monoclonal antibodies either gave no or very weak staining, or showed non-specific staining. Remarkably, two different antigen staining patterns were noticed. Monoclonal IGH 222, directed against E2, clearly stained hepatocytes (Figure 1), while IGH 207 and IGH 210, directed against E1, stained lymphocytes infiltrating in the liver (Figures 2a and 2b). IGH 207 also stained hepatocytes, but

to a weaker degree than IGH 222 (compare Figures 1 and 2a). This staining pattern was confirmed on a series of biopsies of five different patients.

Monoclonal	envelope	staining pattern
IGH 200	E1	negative
IGH 201	E1	weak
IGH 202	E1	negative
IGH 204	E1	weak
IGH 207	E1	strong positivity of lymphocytes, weaker positivity of hepatocytes
IGH 209	E1	negative
IGH 210	E1	positive on lymphocytes (only noted after formaldehyde fixation)
IGH 212	E2	weak
IGH 214	E2	negative
IGH 215	E2	negative
IGH 216	E2	negative
IGH 219	E2	negative
IGH 221	E2	negative
IGH 222	E2	strong positivity of hepatocytes

Example 3: Identification of monoclonal antibodies allowing detection of viral envelope antigen in peripheral blood cells.

The finding that lymphocyte infiltrates in the liver can be stained for HCV envelope antigens prompted us to look also at peripheral blood mononuclear cells. In order to allow intracellular staining, peripheral blood cells were permeabilised with saponin, allowed thereafter to react with the monoclonal antibodies IGH 201 or 207 (directed against E1 and showing weak and strong positivity on the liver biopsies, respectively). Finally, reactivity was checked on a fluorescent cell sorter using secondary FITC-labelled antibodies. IGH 207, which stained already the lymphocyte infiltrates in the liver, showed a high specificity. With this monoclonal, almost no background staining was detected, and 4 out of 5 patients clearly stained positive (Figure 3). The second monoclonal, IGH 201 (binding to SEQ ID 29; ECACC accession number: 98031216) which is

also directed against E1, yields a higher background but intracellular E1 was detected in 5 out of 5 patients as can be deduced from the histograms presented in Figure 4, which show a clear subpopulation of cells with a higher degree of fluorescence as compared to the control sample.

Example 4: Mapping of the reactive monoclonal antibodies against E1 or E2

All 14 monoclonal antibodies were mapped to their respective epitopes using peptides scanning the E1 and E2 protein against which the antibodies were raised. These peptides were biotinylated, bound to streptavidin sensitized microtiterplates, and allowed to react with the monoclonal antibodies. As a positive control recombinant envelope proteins were checked.

For each monoclonal antibody reactivity could be assigned to a specific epitope region defined by two overlapping peptides (for details on sequences see Table I).

peptides	aa region	reactive monoclonals
V1V2	192-226	IGH 201, 204, 202, 200
V2V3	212-244	IGH 201, 204, 202, 200
V3V4	230-263	
HR	261-290	
V5C4	288-327	IGH 207, 210, 209
C4V6	307-340	IGH 207, 210, 209
recombinant	192-326	IGH 201, 207, 210, 204, 202, 200, 209
HVR I	384-415	IGH 222, 215
HVR1/C1a	395-428	IGH 222, 215
C1a	413-447	
C1b	430-467	
HVRII	460-487	IGH 212, 214, 221
C2a	480-513	IGH 219, 216
C2b	500-530	
V3-C3	523-566	
V4	561-590	
C4a	578-627	

C4b	621-648	
C4c	641-673	
recombinant	384-673	IGH 222, 215, 212, 214, 221, 219, 216

The epitope for IGH 201 can be defined as the region 212-226 (SEQ ID 29), for IGH 207 and IGH 210 this is 307-326 (SEQ ID 30) and for IGH 222 this is 395-415 (SEQ ID 31). The amino acid region of IGH 201 is a rather variable region of the E1 protein of HCV and has already been previously reported in relation to *in situ* detection of HCV (Hiramatsu et al., 1992, Kaito et al., 1994). However, from our studies it is clear that antibodies directed against this epitope are less suitable for *in situ* detection of HCV as the liver biopsy staining with this antibody was negative and the staining on peripheral blood lymphocytes showed considerable background. In contrast, IGH 207 and IGH 210 recognise a completely conserved region of E1 (Maertens and Stuyver, 1997). The monoclonal IGH 222 recognises a region of E2, which is part of the N-terminal hypervariable domain of E2, and proved to be very suitable for efficient *in situ* detection of HCV.

Example 5: Determination of cross-reactivity on variable epitopes

Using an extended series of peptides derived from various sequences of the N-terminal hypervariable epitope of E2, IGH 222 was further characterised. Table 2 shows a summary of these experiments. From this table it can be concluded that this monoclonal reacts with several sequences, but fails to react with some others. Knowing this epitope is sufficient for the man skilled in the art to raise additional antibodies against this epitope with a better reactivity towards other sequences which are not recognised by IGH 222. Such sequences are by way of example the peptides with # 490, 940, 884, 484 and 494 but other sequences in the region between aa 395-415 may be found against which IGH 222 may fail to react.

From these examples it is clear that the epitopes recognised by the monoclonal antibodies IGH 201, 207, 210 and 222 are readily accessible for binding antibodies and allow detection of the antigen in liver biopsies and peripheral blood cells. The properties of the monoclonal antibodies IGH 201, 207, 210 and 222 are rather unique, since other monoclonals directed against the same epitopes resulted in high background and absence of specific staining. For example, the monoclonal antibodies IGH 207, 209 and 210 all recognise the same epitope, however, IGH 209 is not able to stain E1 antigen in any cell type, while IGH 210 only stains lymphocytes and IGH

207 stains both lymphocytes and hepatocytes. With the instant invention, it should be feasible for the man skilled in the art to produce large series of antibodies (either polyclonal or monoclonal of nature, in various species), that can be used for detection of natural HCV protein antigens.

The need to produce a series of antibodies against a given epitope reflects the fact that the behaviour of an antibody is not exclusively determined by the epitope recognised but also by secondary properties such as affinity and avidity for the epitope, solubility, isotype, and the species in which it is generated. For each application an antibody with different secondary properties may be required. Thus, the production of such large series of antibodies will allow to identify some of them as having similar properties as IGH 201, 207, 210 and 222 i.e. to be able to reveal the presence of HCV envelope protein in biological samples of the host. Indeed, the knowledge that these epitopes are clearly accessible on the HCV envelope proteins, as expressed in the host, provides the necessary information to develop assays to detect these antigens not only *in situ* as presented here but also in solution (eg in plasma or serum). The monoclonal antibodies IGH 201, 207, 210 or 222, or others developed against the same epitope but with optimal characteristics for detection of soluble antigen, possibly in combination with others, could also be used to develop such an assay.

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Table 1

E1 peptides	Genotype	name	#	aa
YQVRNSTGLYHVTNDCPNSSIVYEAADAILHTPGC	1a	V1V2	1071	192-226
YEVRNVSGIYHVTNDCSNSSIVYEAADMIMHTPGC	1b	V1V2	888	192-226
VEVKNNSNSYMATNDCSNSSIIWQLEGAVLHTPGC	2a	V1V2	1019	192-226
VEVKNTSTSYMVTNDCSNSSIVWQLEGAVLHTPGC	2c	V1V2	1074	192-226
LEWRNTSGLYVLTNDCSNSSIVYEADDVILHTPGC	3	V1V2	1008	192-226
INYRNVSGIYHVTNDCPNSSIVYEADHHILHLPGC	4	V1V2	1075	192-226
VPYRNASGIYHITNDCPNSSIVYEADNLILHAPGC	5	V1V2	1084	192-226
LTYGNSSGLYHLTNDCSNSSIVLEADAMILHLPGC	6	V1V2	1023/1085	192-226
LNYANKSGLYHLTNDCPNSSIVYEANGMILHLPG	7	V1V2	1334	192-225
IQVKNASGIYHLTNDCSNSSIVFEAETMILHLPGC	9	V1V2	1333	192-226
LEYRNASGLYMVTNDCSNGSIVYEAGDIILHLPGC	10	V1V2	1332	192-226
IVYEAADMIMHTPGCVPCVRENNSSRCWV	1b	V2V3	1036	212-244
VRENNSSRCWVALTPTLAARNASVPTTTIRRHVD	1b	V3V4	1022	230-263
HVDLLVGAAAFCSAMYVGDLCGSVFLVSQL	1b	HR	1150	261-290
SQLFTISPRRHETVQDCNCSIYPGHITGHRMAWDMMMNWS	1b	V5C4	1176	288-327
SIYPGHITGHRMAWDMMMNWSPTTALVVSQLLRI	1b	C4V6	1039	307-340

Table 1 continued

E2 peptides	Genotype	name	#	aa
HTRVSGGAAASNTRGLVSLFSPGSAQKIQLVN	1b	HVR I	1139	384-415
NTRGLVSLFSPGSAQKIQLVNTNGSWHINRTALN	1b	HVR I/C1a	1173	395-428
LVNTNGSWHINRTALNCNDSLQTGFFAALFYKHKF	1b	C1a	1149	413-447
NDSLQTGFFAALFYKHKFNSSGCPERLASCRSIDKFAQ	1b	C1b	1148	430-467
RSIDKFAQGWGPLTYTEPNSSDQRPYCW	1b	HVR II	1020	460-487
SDQRPYCWHYAPRPCGIVPASQVCGPVYCFTPSP	1b	C2a	1147	480-513
SQVCGPVYCFTPSPVVVGTTDRFGVPTYNWG	1b	C2b	1143	500-530
GVPTYNWGANDSDVLILNNTRPPRGNWFGCTWMNGTGFTKTCGG	1b	V3-C3	1178	523-566
TKTCGGPPCNIGGAGNNTLTCPTDCFRKHP	1b	V4	1142	561-590
TDCFRKHPEATYARCGSGPWLTPRCMVHYPYRLWHYPCTVNFTIF	1b	C4a	16	583-627
TVNFTIFKVRMYVGGVEHRFEAACNWTR	1b	C4b	1141	621-648
EAACNWTRGERCDLEDRDRSELSPLLLSTTEWQ	1b	C4c	1140	641-673
KTTNRLVSMFASGPKQNVHLINT	-	HVR I	485	394-416
HTTSTLASLFSPGASQRIQLVNT	-	HVR I	492	395-416
HVTCTLTSLFRPGASQKIQLVNT	-	HVR I	489	394-416
AHNARTLTGMFSLGARQKIQLINT	-	HVR I	520	394-416
SDTRGLVSLFSPGSAQKIQLVNT	-	HVR I	886	394-416
SSTQSLVSWLSQGPSQKIQLVNT	-	HVR I	494	394-416
HTMTGIVRFFAPGPKQNVHLINT	-	HVR I	484	394-416
RAMSGLVSLFTPGAKQNIQLINT	-	HVR I	884	394-416
HVTGTLTSLFRPGASQKIQLVNT	-	HVR I	940	394-416

Table 2

sequence	#	aa region	Recognition by IGH 222
HTRVSGGAAASNTRGLVSLFSPGSAQKIQLVN	1139	384-415	+
KTTNRLVSMFASGPKQNVHLINT	485	394-416	+
HTTSTLASLFSPGASQRIQLVNT	492	395-416	+
AHNARTLTGMFSLGARQKIQLINT	520	394-416	+
SDTRGLVSLFSPGSAQKIQLVNT	886	394-416	+
SST**Q**SLVS**WL**S**Q**GPSQKIQLVNT	494	394-416	-
HT**M**TGIV**RF**FAPGPKQNVHLINT	484	394-416	-
R**AM**SGLVSLFTPGAKQNIQLINT	884	394-416	-
H**V**T**G**TLTSLF**R**PGASQKIQLVNT	940	394-416	-
RTT**Q**GLVSLFS**R**GAKQ**D**IQLINT	490	394-416	-

non-conservative mutations in the peptides not reacting with IGH 222 are shown in bold

CLAIMS

1. Use of antibodies specifically binding to the C-terminal region of the HCV E1 protein (aa 227-383) or the N-terminal region of the HCV E2 protein (aa 384-450) for the preparation of a natural HCV protein antigen detection kit.

2. The use of antibody according to claim 1 wherein said antibody specifically binds to one of the following epitopes: -aa 307-326 of HCV E1 protein (SEQ ID 30)
-aa 395-415 of HCV E2 protein (SEQ ID 31).

3. The use of antibodies according to any of claims 1 or 2 wherein said antibody is a monoclonal antibody.

4. The use of antibodies according to any of claims 1 to 3 wherein said antibody is secreted by ECACC deposit having accession number 98031214 or 98031215.

5. The use of a functionally equivalent variant or fragment of an antibody according to any of claims 1 to 4.

6. The monoclonal antibody secreted by the ECACC deposit having accession number 98031214.

7. The functionally equivalent variant or fragment of the antibody according to claim 6.

8. The hybridoma cell line of ECACC deposit having accession number 98031214

9. Method for the detection of natural HCV protein antigens comprising:
-contacting a test sample which may contain HCV protein antigens with an antibody according to any of claims 1 to 4 or with a functionally equivalent variant or fragment of said antibody, to form an antibody-antigen complex, and
-determining said antigen-antibody complex with an appropriate marker.

10. The method according to claim 9, wherein said test sample comprises human cells or tissues.

11. The method according to claim 10, wherein said human cells are peripheral blood cells.

12. The method according to claim 10, wherein said human tissue is liver tissue.

13. An assay kit for the detection of natural HCV protein antigens comprising:

-an antibody according to any of claims 1 to 4, or a functionally equivalent variant or fragment of said antibody, and

-appropriate markers which allow to determine the complexes formed between HCV protein antigens in a test sample with said antibody or a functionally equivalent variant or fragment of said antibody.

