

657497
AUSTRALIAP/00/001 28/6/91
Section 20

Patents Act 1990

PATENT REQUEST: STANDARD PATENT/PATENT OF ADDITION

We, being the person(s) identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification.

Full application details follow.

[71] Applicant: BEHRINGWERKE AKTIENGESELLSCHAFT
Address: D-3550 Marburg, Federal Republic of Germany

[70] Nominated Person: BEHRINGWERKE AKTIENGESELLSCHAFT
Address: D-3550 Marburg, Federal Republic of Germany

[54] Invention Title: HCV-SPECIFIC PEPTIDES, AGENTS THEREFOR AND THE USE THEREOF

[72] Name(s) of actual inventor(s): UDO KRUPKA, WERNER STUBER, MANFRED GERKEN and STEFAN BRUST

[74] Address for service in Australia: c/o WATERMARK PATENT & TRADEMARK ATTORNEYS, of
The Atrium, 290 Burwood Road, Hawthorn, Victoria 3122, Australia Attorney Code: WM

BASIC CONVENTION APPLICATION(S) DETAILS

[31] Application Number	[33] Country	Country Code	[32] Date of Application
P40 34 982.9	Germany	DE	3rd November 1990
P41 12 743.9	Germany	DE	19th April 1991
P41 20 281.3	Germany	DE	19th June 1991
P41 21 431.5	Germany	DE	28th June 1991

Drawing number recommended to accompany the abstract

By my/our Patent Attorneys,
WATERMARK PATENT & TRADEMARK ATTORNEYS


D. B. Mischlewski

.....31st October 1991.....
(Date)

Registered Patent Attorney

AUSTRALIA

Patents Act 1990

NOTICE OF ENTITLEMENT
(To be filed before acceptance)

We, **BEHRINGWERKE AKTIENGESELLSCHAFT** of, D-3550 Marburg, Germany being the applicant in respect of Application No. 87002/91 state the following:-

The Person nominated for the grant of the patent has entitlement from the actual inventors by virtue of an employment contract.

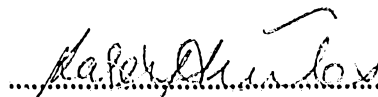
The person nominated for the grant of the patent is the applicant of the basic application listed on the patent request form.

The basic application listed on the request form is the first application made in a Convention country in respect of the invention.

BEHRINGWERKE AKTIENGESELLSCHAFT

By our Patent Attorneys,

WATERMARK PATENT & TRADEMARK ATTORNEYS


.....

Karen J. Sinclair

Registered Patent Attorney

3 January 1995
.....



AU9187002

(12) PATENT ABRIDGMENT (11) Document No. AU-B-87002/91
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 657497

- (54) Title
HCV-SPECIFIC PEPTIDES, AGENTS THEREFOR AND THE USE THEREOF
- (51)⁵ International Patent Classification(s)
C07K 007/10 A61K 039/21 A61K 039/29 C07K 007/06
C07K 007/08 C07K 015/04 C07K 015/12 C07K 017/14
C12N 015/51 G01N 033/68 G01N 033/569 G01N 033/576
- (21) Application No. : 87002/91 (22) Application Date : 01.11.91
- (30) Priority Data
- | (31) Number | (32) Date | (33) Country |
|-------------|-----------|--------------|
| 4034982 | 03.11.90 | DE GERMANY |
| 4112743 | 19.04.91 | DE GERMANY |
| 4120281 | 19.06.91 | DE GERMANY |
| 4121431 | 28.06.91 | DE GERMANY |
- (43) Publication Date : 07.05.92
- (44) Publication Date of Accepted Application : 16.03.95
- (71) Applicant(s)
BEHRINGWERKE AKTIENGESELLSCHAFT
- (72) Inventor(s)
UDO KRUPKA; WERNER STUBER; MANFRED GERKEN; STEFAN BRUST
- (74) Attorney or Agent
WATERMARK PATENT & TRADEMARK ATTORNEYS , Locked Bag 5, HAWTHORN VIC 3122
- (56) Prior Art Documents
AU 646275 81390/91 C07K 7/10 A61K 37/02
AU 74399/91 C07K 7/06 A61K 37/02 C12P 21/08
AU 68390/90 G01N 33/576 C07K 7/08
- (57) The invention also relates to an immunochemical method for the simultaneous detection and/or for the simultaneous determination of a plurality of different antibody specificities against pathogens which are different in each case in a single test.

CLAIM

1. A mixture of peptides composed of at least one of the peptides of group A which group consists of the peptides:

1	30
MSTNPKPQRKTKRNTNRRPQDVKFPGGGQI	SP 10
8	26
QRKTKRNTNRRPQDVKFPG	SP 23
10	26

(11) AU-B-87002/91
(10) 657497

-2-

KT	KR	NT	NR	RP	QD	VK	FPG		SP 30
12						26			
KR	NT	NR	RP	QD	VK	FPG			SP 30 D
14						26			
		NT	NR	RP	QD	VK	FPG		SP 30 C
16						26			
		NR	RP	QD	VK	FPG			SP 30 B
18						26			
				RP	QD	VK	FPG		SP 30 A
8						24			
QR	KT	KR	NT	NR	RP	QD	VKF		SP 31
8						22			
QR	KT	KR	NT	NR	RP	QDV			SP 32

and at least one of the peptides of group B (non-structural protein of HCV), which group consists of the peptides:

4083:

121

SGKPAIIPDREVLVYREFDEMEECSQHLPYIEQGMMMLAEQFKQKALGLLQTASRQA

4074:

121

160

SGKPAIIPDREVLVYREFDEMEECSQHLPYIEQGMMMLAEQF

4073:

128

160

PDREVLVYREFDEMEECSQHLPYIEQGMMMLAEQF

4072:

136

160

EFDEMEECSQHLPYIEQGMMMLAEQF

(11) AU-B-87002/91
(10) 657497

3

4071:

144 160
SQHLPYIEQGMMMLAEQF

4081:

161 175
KQKALGLLQTASRQA

4082:

144 175
SQHLPYIEQGMMMLAEQFKQKALGLLQTASRQA

4090:

135 164
REFDEMEECSQHLPYIEQGMMMLAEQFKQKA

4056:

121 135
SGKPAIIPDREVLRY

4055:

129 143
DREVLRYREFDEMEEC

4054:

137 151
FDEMEECSQHLPYIE

4053:

145 159
QHLPYIEQGMMMLAEQ

4052:

153 167
GMMMLAEQFKQKALGL

4060:

121 139
SGKPAIIPDREVLRYREFDE

AUSTRALIA

Patents Act 1990

65 7497

**ORIGINAL
COMPLETE SPECIFICATION
STANDARD PATENT**

Application Number:

Lodged:

Invention Title: HCV-SPECIFIC PEPTIDES, AGENTS THEREFOR AND THE USE THEREOF

The following statement is a full description of this invention, including the best method of performing it known to :- us

5

Description

HCV-specific peptides, agents therefor and the use thereof

10

The invention relates to polypeptides for the immunochemical determination of HCV-specific antibodies and HCV antigens, and to agents suitable for this method and the use thereof.

15

The invention also relates to an immunochemical method for the simultaneous detection and/or for the simultaneous determination of a plurality of different antibody specificities against pathogens which are different in each case in a single test.

20

Non A/non B hepatitis (NANBH) is, as transmissible disease or group of clinical pictures, regarded as virus-associated and distinguishable from other virus-induced clinical pictures which are caused by different hepatitis viruses such as hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis D virus (HDV) and hepatitis E (HEV). Finally, it is also possible to diagnose hepatitis caused by cytomegalovirus (CMV) or Epstein-Barr virus (EBV).

30

It is possible on the basis of epidemiological investigations to define, in accordance with the transmission route, at least two types of non A/non B hepatitis (NANBH): epidemic hepatitis virus (enterically transmitted NANBV), which is transmitted by water and foodstuffs, and post-transfusion hepatitis virus (blood transmitted NANBV), which is transmitted by blood, needle sticks or similar routes. Besides these routes of infection there are also known to be transmissions which, as

35

sporadically occurring NANBV ("community acquired NANBV"), have no evident association with the two types mentioned. Although the exact number of agents or viruses causing NANBH is unknown, so-called hepatitis C virus (HCV) has recently been identified as a causative pathogen of this disease (WO 89/04 669).

Until recently, clinical diagnosis was based mainly on serological determination of antigens and/or on antibodies directed against them, the tests being specific for parameters from the group of hepatitis pathogens HAV, HBV, HDV, HEV, CMV or EBV. In this so-called exclusion method, NANBH was diagnosed only when all the abovementioned determinations were negative.

Besides this, so-called surrogate markers have also been used, such as, for example, GPT (glutamic-pyruvic transaminase, also called ALT - alanine aminotransferase) or anti HBc (hepatitis B core-specific antibodies). However, these aids are neither sensitive nor specific enough to be regarded as reliable. It is therefore possible to avoid only a small part of the cases of post-transfusion hepatitis, which occur in about 10% of transfused patients, by investigating blood donors. The urgent need for introduction of a specific test is underlined by the fact that NANBV is regarded as responsible for about 90% of cases of post-transfusion hepatitis. The main problem with this disease is the fact that between 25 and 55% of infected people suffer chronic liver damage.

The discovery of HCV has provided the basis for a specific detection of HCV or HCV-specific antibodies. The isolation and characterization of HCV and corresponding cDNA replicates of parts of the HCV genome is the subject of WO 89/04 669, where HCV is assigned to the family of flavi-like viruses. There are also descriptions therein of the use of HCV antigen for detecting HCV-specific antibodies, and of the raising of antibodies for the diagnostic determination of antigen in patient's blood.

However, use is made of genetically engineered proteins, especially so-called non-structural proteins (NSP) from the open reading frame region (ORF), which are used as reactants in immunochemical detection methods.

5 An immunochemical detection comprises within the meaning of this invention all processes which, as homogeneous (in solution) or heterogeneous (with solid phase) in vitro methods, permit the determination of antigens and/or antibodies of immunoglobulin classes A, D, E, G or M
10 (IgA, IgD, IgE, IgG or IgM) in body fluids such as serum, plasma, saliva, cerebrospinal fluid or urine. Examples of these methods, which are also called immunoassay, are enzyme immunoassay (ELISA or EIA), radioimmunoassay (RIA), immunofluorescence assay (IFA), radioimmunoprecipitation (RIPA), agar gel diffusion etc.
15

For example, WO 89/04 669 makes use of the antigen C-100-3, mentioned therein, in the ELISA procedure for detecting HCV-specific antibodies (anti-HCV). The construct C-100-3, which is expressed by genetic engineering in yeast cells, from the NSP 3/4 region comprises 363 amino acids, whose sequence is depicted in Fig. 1, where the numbering system corresponds to that in the abovementioned patent.
20

However, the maximum sensitivity which can be achieved with the ELISA method for determining anti-HCV in samples of human origin is assumed to be about 80% in the case of chronic NANB patients and about 30% in the case of acute NANB patients. These findings on blood from human patients are supported by corresponding investigations on chimpanzees infected with NANBV.
25

30 In them, positive anti-HCV detection on the basis of the C-100-3 construct does not succeed until about 6 - 18 weeks after the ALT increase has taken place, which in turn can be observed as sign of disease 3 - 10 weeks after inoculation of NANBV. Thus, it emerges that 9 - 26
35 weeks after infection of a chimpanzee is the total time

before HCV-specific antibodies can be detected with the immunoassay according to the state of the art using the C-100-3 construct.

5 WO 90/11 089 describes further HCV amino acid sequences which can be used to detect anti-HCV. However, no statements are made about the diagnostic relevance of particular protein sections, nor are there examples of immunodominant epitopes.

10 The fundamental problem in the detection of anti-HCV with the methods disclosed in the literature is that there are still samples which react in a false-positive and false-negative way. The false-positive samples may comprise up to 40% in a group of healthy blood donors (WEINER A., et al. Lancet 1990, Vol. 336, p. 695). The lack of a specific and sensitive HCV test therefore means that a not inconsiderable proportion of samples which react falsely are wrongly discarded in blood donation centers and, at the same time, patients who are actually anti-HCV-positive are still not reliably identified.

20 To eliminate these disadvantages, other groups have selected particular sequences of the ORF region of C-100-3 (from WO 89/04 669, Fig. 1) and employed them in an immunoassay (Peptides sp42, 117, 67 and 65, Fig. 2). On investigation of NANB-infected chimpanzees it was not possible to achieve an improvement in the diagnostic value. On the contrary, the observations made therein led to the conclusion that none of the described peptides is suitable to serve as basis for a reliable IgG or IgM determination, because the sensitivity is inadequate and the diagnostic gap of at least 7 to 17 weeks until antibodies are detected means that there is no reduction in time compared with conventional detection methods. Furthermore, it was found that sp42 in particular, with 30 a diagnostic gap lasting 20 to 40 weeks, is unsuitable for a diagnostic test (SAFFORD J., et al. Int. Symp. on Viral Hepatitis and Liver Disease 1990 Houston, USA).

However, the polypeptide called sp67 is said to be immunodominant although only 86% identification of anti-HCV-positive samples was possible with this peptide (DAWSON, G.J., et al. Int. Congress of Virology 1990, Berlin, FRG).

An improvement is said to have been achieved with synthetically prepared core peptide (OKAMOTO H., et al. Japan. J. Exp. Med. 1990, Vol 60, No. 4, p. 223 - 233). This entailed selection of a sequence from the total of 3 potential antibody binding sites of the core amino-acid sequence 1-120 (OKAMOTO, H. et al., Japan. J. Exp. Med. 1990, Vol. 60, No. 3, p. 167 - 177) with the aid of physicochemical predictive criteria specified by HOPP and WOOD (Proc. Nat. Acad. Sci. 1981, Vol. 78, p. 3824 - 3828). This peptide is depicted in Fig. 3 and comprises 36 amino acids numbered from No. 39 to No. 74.

In contrast to the anti-C-100 test, it was possible to detect HCV antibodies in some anti-HCV-positive sera with an ELISA based on this core peptide. This finding was confirmed by a comparative investigation using the polymerase chain reaction (PCR) to detect HCV RNA.

However, it is evident from the 26 anti-core HCV-positive samples found in an investigation on 606 blood donors (4.3%) precisely that only 10 (1.65%) cases could be confirmed by PCR. Conversely, 16 donors (2.65%) gave false positive reactions with this peptide. Thus, although the said core peptide is said to be more suitable in some cases for an anti-HCV detection method than the anti-C-100 test, the core peptide of the state of the art is generally associated with considerable susceptibility to interference.

The present invention was thus based on the object of developing a test able to detect HCV infections as early as possible and with high specificity.

5 It has now been found, surprisingly, that certain polypeptides from the area of the ORF region of C-100-3 and/or the amino-terminal region of the HCV core region are particularly suitable for detecting HCV-specific antibodies, and a distinct increase in sensitivity compared with peptides of the state of the art with, at the same time, significantly reduced susceptibility to interference from unwanted false positive reactions can be achieved.

10 It has furthermore been found that a high antigen concentration, and thus a high epitope density, is achieved in an immunochemical detection method with the peptides according to the invention. This is possible particularly when, owing to the absence of interfering contaminations, the investigation of highly concentrated patients' samples is possible.

15 It was also completely surprising that the peptides according to the invention carry immunodominant epitopes for early and late HCV antibodies and thus can be employed particularly advantageously for longitudinal investigations.

20 Hence the invention relates to peptides or peptide mixtures which react specifically with antibodies against an HCV infection, the amino-acid sequences thereof containing parts of the ORF region of C-100-3 and/or of the amino-terminal HCV core region.

The terms peptides and polypeptides are used within the scope of the invention as equivalent to peptides with up to about 80 AA.

30 The peptides according to the invention preferably comprise amino-acid sequences from AA 121 to AA 175 (formula I) and from AA 337 to AA 363 (formula II) of the HCV genome section described as C-100-3, with the following sequences:

121

175

SGKPAIIPDREVLVREFDEMEEC SQHLPYIEQGMMMLAEQFKQKALG LLQTASRQA I

337

363

HVGPGEGAVQWMNRLIAFASRG NHVSP

II

Bevorzugte Polypeptide sind:

Preferred polypeptides are:

4083:

121

175

SGKPAIIPDREVLVREFDEMEEC SQHLPYIEQGMMMLAEQFKQKALG LLQTASRQA

4074:

121

160

SGKPAIIPDREVLVREFDEMEEC SQHLPYIEQGMMMLAEQF

4073:

128

160

PDREVLVREFDEMEEC SQHLPYIEQGMMMLAEQF

4072:

136

160

EFDEMEEC SQHLPYIEQGMMMLAEQF

4071:

144

160

SQHLPYIEQGMMMLAEQF

4081:

161

175

KQKALG LLQTASRQA

4082:

144

175

SQHLPYIEQGMMMLAEQFKQKALG LLQTASRQA

4090:

135

164

REFDEMEEC SQHLPYIEQGMMMLAEQFKQKA

4056:

121 135
SGKPAIIPDREVLJR

4055:

129 143
DREVLJREFDEMEEC

4054:

137 151
FDEMEEC SQHLPYIE

4053:

145 159
QHLPYIEQGMMMLAEQ

4052:

153 167
GMMMLAEQFKQKALGL

4091:

345 359
VQWMNRLIAFASRGN

Sequences from the carboxyl- and amino-terminal region of formula I in particular have proven beneficial for detecting late antibodies of an HCV infection, such as, for example, the peptides 4081, 4056, 4055, and 4060 hereinafter:

4060:

121

139

SGKPAIIPDREVLVREFDE

Surprisingly, the carboxyl-terminal region of the ORF region of C-100-3 has proven particularly suitable for detecting late antibodies, containing amino acids of the formula II or parts thereof, for example 4091.

5

It has also been found that the central region of formula I is relevant for early identification of anti-HCV. Preferred in this connection are the abovementioned polypeptides 4071 and 4072, and peptides 4054, 4053 and 4052.

10

Furthermore 3 epitopes have been located on the peptide of formula I, one of which recognises a late antibody (S) and the others recognise early antibodies (F1 and F2). Examples of preferred peptides have one of the following amino-acid sequences:

15

AA_{a1} -DREVLVYR- BA_{b1} (S)

20

AA_{a2} -QHLPYIE- BA_{b2} (F1)

25

AA_{a3} -KQKALGL- BA_{b3} (F2)

where AA and BA are any desired amino acid, and $a1 - a3$ and $b1 - b3$ are each, independently of one another, integers greater than or equal to zero.

It is advantageous to select the amino-terminal sequences together with the carboxyl-terminal amino-acid sequences of the formula I and the sequence of the formula II. The central amino-acid sequence of the formula I from about 130 to 160 can be used separate therefrom.

The peptides according to the invention furthermore comprise parts of the amino-terminal HCV core region, preferably that amino-acid sequence from AA 1 to AA 35 of the HCV genome section described as core, with the following sequence:

MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVY (III)

Preferred peptides are:

1	30	
MSTNPKPQRKTKRNTNRRPQDVKFPGGGQI		SP 10
8	26	
QRKTKRNTNRRPQDVKFPG		SP 23
10	26	
KTKRNTNRRPQDVKFPG		SP 30
12	26	
KRNTNRRPQDVKFPG		SP 30 D
14	26	
NTNRRPQDVKFPG		SP 30 C
16	26	
NRRPQDVKFPG		SP 30 B
18	26	
RPQDVKFPG		SP 30 A
8	24	
QRKTKRNTNRRPQDVKF		SP 31
8	22	
QRKTKRNTNRRPQDV		SP 32

The peptides SP 10 and SP 23 are particularly preferred.

It is possible with the aid of the peptides according to the invention of the core region to identify both early antibodies from acute phases of infection and late antibodies, which considerably improves the sensitivity of the detections based on these peptides compared with the state of the art. Another advantage is the high specificity of the peptides, which generally results in a minimal number of samples with a false positive reaction.

It is generally advantageous to use peptides, linked peptides or peptide mixtures which are specific for early and late HCV antibodies, because it is possible with both types of binding site to identify both samples from early and samples from late phases of infection. It is also possible reliably to identify seroconversions caused by HCV-specific IgG and/or IgM antibodies, and it is generally possible to discriminate between positive and negative samples with extremely high accuracy. In addition, there is generally no interference by the expression system required for protein preparation by genetic engineering, or other host cell contaminations which are unavoidable for growing viruses. Reliable determination of HCV-specific antibodies is also generally ensured in sera, citrated, heparinized or EDTA plasma of human origin, and inactivation of the samples at about 56°C for about 60 minutes generally gives rise to no false positive results. Finally, these peptides according to the invention can be used to prepare specific antibodies with whose aid, owing to the immunochemical determination of corresponding antigens in cell-free patient's blood, the diagnostic gap can be further closed.

According to the subject-matter of the invention, a series of novel peptides which preferably identify HCV-specific antibodies from convalescent or chronically infected patients and/or HCV antibodies from acute phases of infection are described, and polypeptides and mixtures

of polypeptides which are suitable as basis for screening tests for the non-differentiating detection, which is, however, highly sensitive and little susceptible to interference, of anti-HCV antibodies are described.

5 The invention furthermore relates to antibodies which have a biospecific affinity for at least one of the peptides, described above, of the ORF region of C-100-3 or of the core region.

10

The immunochemical determination of corresponding antigens with the antibodies according to the invention in cell-free patient's blood makes it possible to establish the presence of HCV even before endogenous antibodies appear.

15

The invention furthermore relates to an immunochemical method for the detection and/or for the determination of HCV antibodies with the peptides according to the invention as antigen.

20

The invention also relates to a method for the purpose of differential diagnosis between an early and late phase of infection, where in each case one or more peptides which react specifically to early antibodies and one or more peptides which react specifically to late antibodies are reacted with the sample in separate mixtures.

25

The invention furthermore relates to the use of the peptides described above for raising antibodies in mammals, especially in humans.

The invention also relates to the use of the antibodies described above for diagnostic and therapeutic purposes.

30

The invention therefore further relates to agents which contain at least one of the antibodies or peptides according to the invention alone or in combination with other peptides or antibodies.

The said immunoreactive peptides can be prepared by synthesis or genetic engineering, preferably by synthesis by the methods known to the person skilled in the art. The chemical synthesis of the peptides can be carried out, for example, as described by BARANI, G. and MERRIFIELD, R.B. in "The Peptides, Analysis, Synthesis and Biology", Vol. 2, Academic Press 1980, ed. Erhard Gross, Johannes Meyenhofer, especially as a polypeptide or as mixture of several small peptides with overlapping or non-overlapping amino-acid sequence.

The polypeptides prepared by genetic engineering include fusion proteins whose fusion portion has subsequently been eliminated. Also included are polypeptides which have been modified where appropriate, for example by glycosylation, acetylation or phosphorylation.

These amino-acid sequences can be synthesized both as a polypeptide and as mixture of several small peptides with overlapping or non-overlapping amino-acid sequence.

It has also been found that mixtures of individual peptides may have better diagnostic properties for an immunochemical anti-HCV detection than single peptides of the said structures. It is particularly advantageous to use mixtures of peptides of the ORF region of C-100-3 and of the core region.

The invention therefore further relates to mixtures of peptides containing one or more of the peptides according to the invention.

In another embodiment, 2 or more of the said peptides, preferably 2 to 10, especially 2 to 4 peptides are linked with or without bridge. The length of the peptides is preferably 6 to 15 amino acids. It is even possible for polymeric forms of two or more peptides to be prepared by methods known to the person skilled in the art and bound to a carrier such as, for example, protein or latex

particles. Thus, particularly suitable as carrier or bridge are, for example, human serum albumin and/or polylysine. It is likewise possible to modify the peptides by an extension with 1 to 40, preferably 1 to 20, in particular 1 to 10 amino acids. The additional regions and structures may have a beneficial effect on the physicochemical behavior of the complete peptide, but the immunoreactivity of the peptides or parts thereof ought to be retained.

10 The invention therefore also relates to peptides which are linked to one another with or without bridge, or can also be bound to a carrier.

Modifications of this type generally alter the passive adsorption or covalent bonding property to the solid phase in a beneficial manner, have an advantageous effect on the coupling method or act more strongly as antigen when raising polyclonal or monoclonal antibodies directed against the peptides.

Thus, for example, the invention also relates to peptides of the following formula



where AA and BA are any desired amino acid, and n and m are each, independently of one another, integers from 0 to about 60, in particular from 1 to 40.

25 It is often advantageous for peptides to be derivatized in a variety of ways, such as, for example, by amino-terminal or carboxyl-terminal attachment of one or more amino acids, preferably cysteine, in order, for example, to achieve the linkage of peptides with one another or to
30 a carrier, thioglycolic acid amidation, carboxyl-terminal amidation such as, for example, with ammonium or methylamine. Modifications of this type may alter the net charge on the polypeptide and improve the physicochemical

properties of the peptide or facilitate covalent bonding of the peptide to a solid carrier, to carrier proteins or to another peptide. The person skilled in the art is also aware that the peptides according to the invention can also be prepared among themselves or with themselves by genetic engineering or synthesis in such a way that a plurality of immunorelevant epitopes is located on one peptide.

Thus the invention furthermore relates to peptides with an amino-acid sequence according to the invention which has been modified by replacement, addition or deletion of one or more amino acids.

In general, modifications of this type do not result in direct alterations of the immunoreactivity of a peptide, but it is perfectly possible to achieve improved immunological properties of the peptides. Thus, for example, methionine is prone to spontaneous oxidation, which can be prevented by replacement by norleucin without essentially changing the antigenic properties of the polypeptide.

The person skilled in the art is aware that given amino-acid sequences can be subjected to a wide variety of alterations such as, for example, deletions, insertions or substitutions, which may be associated with various advantages. Modifications of this type relate, for example, to combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; Phe, Tyr; Ala, Ser; Ala, Thr; Ala, Val; Ala, Pro; Ala, Glu; Leu, Gln; Gly, Phe; Ile, Ser and Ile, Met.

It may likewise be advantageous to improve the adsorption properties of the polypeptide in the form of addition of a hydrophobic sequence comprising about 2 to 20 hydrophobic amino acids, such as, for example, Phe Ala Phe Ala Phe.

The invention furthermore relates to DNA sequences which code for at least one of the peptides according to the invention.

5 The invention also relates to an analytical method for the detection and/or for the determination of HCV, employing as specific step a hybridization reaction in which at least one nucleic acid probe which is complementary in its specific part with at least one of the DNA sequences according to the invention is used.

10 An immunochemical detection generally comprises processes which, as homogeneous (in solution) or heterogeneous (with solid phase) methods, permit the determination of antigens and/or antibodies. Examples of these, which are also called immunoassays, are enzyme immunoassay (ELISA or EIA), radioimmunoassay (RIA), immunofluorescence assay (IFA), radioimmunoprecipitation assay (RIPA) or agar gel
diffusion assay etc.

These numerous, very diverse methods differ in specific embodiments in the marker used for detection or the measurement principle (for example photometric, radio-
metric, visual or by the aggregation, scattered light or precipitation behavior) and in the solid phases. The person skilled in the art is aware that separation of bound and free sample antibodies or antigen is, although
widely used, not absolutely necessary, such as, for example, in so-called homogeneous assays. Heterogeneous immunoassays are preferred, especially heterogeneous ELISA methods.

30 The person skilled in the art is likewise aware that the term "confirmatory test" describes only the use of an immunoassay, similar to the way that the tests called dot methods only describe by name the way in which the antigen is immobilized.

35 In the antibody detection, it is a prerequisite for an immunochemical detection method that there is contact of

the sample with the described peptide sequences during the course, in order to form an antigen-antibody complex in a particular step of the particular method or, in competition and inhibition tests, to prevent the formation thereof by adding suitable labeled reagents.

In the direct method it is possible for the antibodies to be contacted with peptides bound to solid phases or with labeled peptides or with both, it being immaterial whether the fundamental method is, as 1-, 2- or multi-step method, based on the principle of the 2nd antibody test or the immunometric test design (double antigen sandwich) either with identical or different peptides (or peptide mixtures) on the solid phase and as liquid reagent for the detection and in combination with specific so-called capture antibodies (for example anti-IgM) or affinity reagents (for example protein A).

The peptides can be bound to the solid phase covalently, by adsorption or using specific antibodies or similar affinity methods, for example via the biotin/avidin complex, but preferably by adsorption.

Suitable as carrier material for the solid phase are plastics such as polystyrene, polyvinyl chloride, polyamide and other synthetic polymers, natural polymers such as cellulose and derivatized natural polymers such as cellulose acetate and nitrocellulose, as well as glass, especially as glass fibers. Polystyrene is preferred as carrier material.

The carriers can be in the form of beads, rods, tubes and microtiter plates or in the form of suspensions such as, for example, latex particles. Sheet-like structures such as strips of paper, small plates and membranes are likewise suitable. The surface of the carriers can be both permeable and impermeable for aqueous solutions.

Preferred carriers are beads, tubes, wells, micro-

particles, strips of paper and membranes. Particularly preferred carriers are microtiter plates, latex particles, polystyrene beads or particles amenable to magnetic attraction.

5 The peptide concentration for coating the carrier is generally about 0.01 - 20 $\mu\text{g/ml}$, preferably 0.01 - 10 $\mu\text{g/ml}$, particularly preferably 2 - 10 $\mu\text{g/ml}$. It is particularly advantageous to use synthetically prepared polypeptides whose high purity and strong antigenicity
10 allow the use of tiny amounts of, for example, 0.01 - 2.0 $\mu\text{g/ml}$, preferably 0.1 - 0.5 $\mu\text{g/ml}$. The binding capacity of the carrier, in particular when polystyrene is used, is generally not saturated so that it is normally possible to coat with a plurality of different polypeptides, in particular with 2 - 5, especially with
15 3 - 4 different polypeptides, which is a particular advantage.

When the peptides are used as labeled derivatives for the detection, suitable coupling techniques are all those
20 known to the person skilled in the art. It is also possible to arrange multistage methods such as, for example, preformed peptide-antibody complexes in which the antibody carries the label, or high affinity systems such as, for example, biotin/avidin with labeling of one
25 of these reactants.

Examples of markers which can be used are radioactive isotopes, fluorescent or chemiluminescent dyes. It is also possible to use enzymes which are detected, for example, by chromogenic, luminogenic or fluorogenic
30 substrate systems, or by subsequent amplification systems with a second enzyme which is activated by the first, as markers.

Preferably used as markers are enzymes, especially alkaline phosphatase and/or horseradish peroxidase or
35 chemoluminogens such as, for example, acridinium esters.

The labeling is carried out by methods which are described for the said markers in the state of the art.

Where the antibodies are labeled with peroxidase, the periodate technique of NAKANE et al., 1974, J. Histochem. Cytochem. 22, 1084 - 1090, can be used, or a method of
5 ISHIKAWA et al., 1983, J. Immunoassay 4, 209 - 327, in which the partners are linked by a heterobifunctional reagent.

Besides these methods, the peptides can also be used for
10 sensitization of suitable surfaces such as, for example, latex or erythrocytes in order to measure, automatically or visually, physicochemical changes induced by peptide-specific antibodies, such as, for example, precipitations, aggregation or light scattering. It is known that
15 the peptides can also be employed not derivatized for inhibition of these measurement principles like also the methods mentioned before.

It is possible to use for detection of the antigens immunodiagnostic methods which make use of polyclonal or
20 monoclonal antibodies which are prepared with the aid of the peptides or derivatives thereof according to the invention. The embodiments suitable for the detection method are known to the person skilled in the art and comprise forming antibody-antigen complexes in a
25 particular step or inhibiting the complex formation in a competition method by adding a labeled antigen.

Suitable as solid phases, markers or measurement principle for establishing an antigen test are all the possibilities described for the corresponding antibody
30 determination, with the competition principle and the double antibody sandwich technique being particularly preferred as immunochemical method. It is immaterial in this context whether the methods are designed as 1-, 2- or 3-step methods. Thus, multistep methods can be carried
35 out with unlabeled detecting antibodies which are

5 determined with the aid of another antibody which is directed against them and is appropriately labeled. It is advantageous for the raising of the antibodies to modify the peptides in such a way that their immunogenic property is improved, as is possible, for example, by coupling to serum albumin or keyhole limpet hemocyanin (B.S. Schaffhausen in Hybridoma Technologie in the Biosciences and Medicine, ed. T.A. Springer, Plenum Press NY, London, 1985).

10 Finally, the present invention can also be applied when using an immunodiagnostic element which contains the solid phase and, in dry form, a part or even all the reagents required, where in this case too the novel peptides are contained either on the side of the solid phase or in the detection reagent or in both, and an antibody determination, an antigen detection or combinations with other analytes is carried out.

20 An unexpected advantage of the novel peptides of the present invention is that they allow reliable determination of HCV antibodies. Furthermore, early antibodies from acute phases of infection are also identified with the aid of these peptides, which considerably improves the sensitivity of the detections based on these peptides compared with the state of the art and, moreover, allows differentiation between acute phases on the one hand and chronic or convalescent stages on the other hand when the peptides with the appropriate binding sites for these antibody types (early or late antibodies) are used separately from one another in two different test methods. Finally, another advantage which emerges is the high specificity of the peptides of the present invention, which leads to a minimization of the number of samples with a false positive reaction.

35 In view of the virus safety in blood donation centers on the one hand and for reasons of cost on the other hand, so-called combination tests have been developed and,

available since 1989, permit simultaneous non-differen-
tiating detection of anti-HIV 1 and/or anti-HIV 2
(K. Körner et al., Lab.Med. 14, 159 - 161, 1990). This
development was possible owing to the great similarity
which the two HIV sub-types display towards one another
and, moreover, they belong to the same virus class. Thus
the anti-HIV 1 determination in such anti-HIV 1/2 com-
bination tests is also based on a cross-reactivity of
anti-HIV 1 with HIV 2 antigens (M. Busch et al., Trans-
fusion 30/2, 184 - 187, 1990), as also conversely the
anti-HIV 2 detection is enhanced by the reactions which
take place between anti-HIV 2 and HIV 1 antigens. On this
basis the establishment of a combined detection of two
antibody specificities proves to be relatively straight-
forward when antigens which are related or structurally
analogous are used. The detection of a pathogen of non A
non B hepatitis, the so-called hepatitis C virus (HCV),
was the prerequisite for establishing an anti-HCV deter-
mination according to the present invention. Despite the
improvement, associated therewith, in the performance
features of screening tests, the problem again arises
also with modern anti-HCV tests that the investigation of
individual donors with anti-HIV combination tests on the
one hand and with anti-HCV single tests on the other hand
entails considerable effort and considerable additional
costs.

All commercial embodiments to date of the anti-HCV
determination and the majority of anti-HIV combination
tests are based on genetically engineered HCV or HIV
polypeptides. However, there has also been no success to
date in determining a plurality of different antibody
specificities in only a single test mixture containing a
plurality of different antigens simultaneously in one
immunochemical detection when it is not possible to use
similar antigens, since different virus class affilia-
tions are present as in the case of HCV (flavivirus) and
HIV (retrovirus). Within the meaning of the invention,
different antibody specificities mean antibodies which

have very low or zero cross-reactivity with one another. It was assumed that a test of this type for detecting a total of three antibody specificities against a plurality of different viral antigens tends to be worse in terms of the sensitivity and in terms of the susceptibility to interference (specificity) as a consequence of mutual interfering interactions than the corresponding properties of the particular single test.

Surprisingly, it has now also been found that a plurality of different antibodies or antibody specificities against different pathogens in each case can be detected immunochemically in a single test when different epitopes of different pathogens in each case are immobilized on a carrier.

Furthermore, the sensitivities found with the method according to the invention at least correspond to the sensitivities of single tests. Thus, for example, the sensitivity features determined for anti-HIV 1/2 and anti-HCV for the method according to the invention at least correspond to those of single tests.

It was therefore also completely surprising that the susceptibility to interference by unwanted false positive reactions was reduced with the method according to the invention. Thus, for example, on testing the specificity of an anti-HIV/anti-HCV test according to the invention the specificity obtained was higher than would be given by the total of the two single tests, with the consequence that overall fewer blood donations may be wrongly discarded.

The invention therefore also relates to an immunochemical method for the detection and/or for the determination of a plurality of different antibody specificities against different pathogens in each case, which comprises one or more epitopes of the particular pathogens being immobilized on a carrier and the detection and/or the

determination of the said pathogens being carried out in a single test.

For certain problems such as, for example, longitudinal investigations, it may be desirable to undertake non-differentiating simultaneous determination of different pathogens. This means that no distinction is made between the various antibodies but only a yes response or no response (negative for all antibody specificities) is obtained.

Also desirable where appropriate is differentiating determination of the simultaneous antibody detection. This is directly possible within the scope of the invention by, for example, in an immunometric test, the antigens which are used having different virus-specific labels, such as, for example, HIV 1 antigens with peroxidase, HIV 2 antigens with alkaline phosphatase and HCV antigens with β -galactosidase. Successive or simultaneous determination of the simultaneously bound antibody specificities is then possible with different enzyme substrates.

An alternative form of differentiation is the inhibition of one antibody specificity by the specific addition of the corresponding antigen to the sample.

The invention thus further relates to an immunochemical method for the simultaneous detection and/or simultaneous determination of different antibody specificities, where the detection and/or the determination is carried out in a differentiating or non-differentiating, preferably non-differentiating, manner.

Different pathogens according to the invention mean pathogens against which antibodies of different specificity and, in general, very low or zero cross-reactivity are directed. Examples of these are HIV 1 + 2, HCV, HTLV I + II, HBV or Treponema pallidum, preferably HIV and HCV.

It is particularly advantageous to immobilize on a carrier antigens of the said pathogens, especially of HIV 1, HIV 2 and HCV, which have a high density or concentration of binding sites (epitopes) for the corresponding antibodies. It is possible in this way, for example, to identify seroconversions, a discrimination of positive and negative samples is possible with an accuracy which is generally high, interference by the expression system which is necessary for preparation of proteins by genetic engineering cannot generally occur, other host cell contaminations which are unavoidable in growing viruses are not generally present, in general reliable determination of HIV- and HCV-specific antibodies with different specificity is ensured in sera, citrated, heparinized and EDTA plasmas of human origin, and inactivation of the samples at 56°C for 60 min normally results in no false-positive results.

Preferably used are single epitopes and/or combinations thereof, especially of polypeptides of HIV 1 and/or HIV 2 and HCV, which are suitable for a highly sensitive anti-HIV/anti-HCV detection, and, furthermore, suitable polypeptide mixtures as basis for a screening test for a combined antibody detection.

The invention thus also relates to polypeptide mixtures of HIV 1 and/or HIV 2 and HCV.

The following polypeptides are particularly preferred:

1. HIV 1 (numbering system of Ratner et al., Nature 1985, 313, 277 - 284):

IV transmembrane protein (gp 41): AA 580 - AA 630

V envelope protein (gp 120): AA 490 - AA 540

VI core protein (p 24): AA 240 - AA 390

2. HIV 2 (numbering system of Gyader et al., Nature 1987, 326, 662 - 669):

VII transmembrane protein (gp 36): AA 570 - AA 620

VIII envelope protein (gp 110): AA 480 - AA 530
IX core protein (p 26): AA 230 - AA 380

3. HCV (numbering system of WO 89/04669 and WO 90/11089):

5 X non-structural protein 4 (NSP 4): AA 121-AA 175
XI non-structural protein 3 (NSP 3): AA 1-AA 265
XII structural protein (core): AA 1-AA 80

10 Especially preferred are mixtures of the said polypeptides, especially for a non-differentiating anti-HIV/anti-HCV screening test, some being described by way of example without confining the conceivable possibilities thereto:

XIII gp 41 HIV 1
gp 36 HIV 2
NSP 3 HCV

XIV gp 41 HIV 1
gp 36 HIV 2
NSP 4 HCV
core HCV

20 XV gp 41 HIV 1
gp 36 HIV 2
NSP 3 HCV
NSP 4 HCV
core HCV

25 XVI gp 41 HIV 1
p 24 HIV 1
gp 36 HIV 2
NSP 3 HCV
core HCV

30 or

XVII gp 41 HIV 1
p 24 HIV 1

gp 36 HIV 2
p 24 HIV 2
NSP 3 HCV
NSP 4 HCV
core

5

In general, better immunological properties for diagnostic use are achieved with the said peptide mixtures.

The following peptides of HIV 1, HIV 2 and HCV have proven particularly suitable:

XVIII SPH 9 (HIV 1, gp 41):

586 620
RILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS

XIX SPH 20 (HIV 2, gp 36):

578 613
RVTATIEKYLQDQARLNSWGCAFRQVCHTTVPWVND

XX SP 4083 (HCV, NSP 4):

121 175
SGKPAIIPDREVLRYREFDEMEECQHLPIYIEQGMMLAEQFKQKALGLLQTASRQA

or parts thereof, for example

XXI a mixture of SP 4060 and SP 4082 (HCV, NSP 4):

121 139
SGKPAIIPDREVLRYREFDE SP 4060

144 175
SQHLPIYIEQGMMLAEQFKQKALGLLQTASRQA SP 4082

XXII SP 10 (HCV, core):

1 30
MSTNPKPQRKTKRNTNRRPQDVKFPGGGQI

and/or

XXIII SP 31 (HCV, core):

8

24

QRKTKRNTNRRPQDVKF-NH₂

It is likewise possible to integrate further peptides from different protein regions of HIV 1, HIV 2 or HCV, as long as these polypeptides are immunorelevant. Conversely, it may also be advantageous, for example for epidemiological problems, to exclude a specific antibody in the non-differentiating detection by omitting corresponding peptides in order, for example, to determine with a mixture of HIV 1 and HCV peptides only anti-HIV 1/anti-HCV simultaneously and not, for example, anti-HBV/anti-HIV 1/anti-HCV. It is also possible for the peptides which are not identical to HCV, such as, for example, precisely HIV, to be derivatized and modified in a manner analogous to that described above for the HCV peptides.

A considerable advantage of the method according to the invention is that a plurality of antibodies against different pathogens can be detected in a single test and, moreover, a specificity or sensitivity which is still as high as that which can be achieved with single tests is achieved.

Fig. 1 Primary sequence of the amino acids of the ORF region of C-100-3 (from WO/89/04669)

Fig. 2 Comparison of the sequences, according to the invention, of the formula I and II with known peptides from the ORF region of C-100-3

Fig. 3 Comparison of the sequences, according to the invention, of the HCV core protein with known peptides

The examples described hereinafter represent embodiments of the invention without, however, confining it to them.

Example 1

Preparation of peptide solutions and coating of micro-titer plates with these peptides or peptide mixtures

Serial 2-fold dilutions in 0.10 M sodium bicarbonate
5 pH 9.6 were made up from stock solutions of the peptides
according to the invention in 50% acetic acid in dis-
tilled water containing 1 to 10 mg of peptide/ml, that
is to say a series with the concentrations 50, 25, 12.5,
6.25, 3.12, 1.56, 0.78, 0.39, 0.2, 0.1, 0.05 and 0.01 μ g
10 of peptide/ml was obtained. The procedure was analogous
for mixtures of single peptides, the stock solutions
additionally being mixed in various ratios, for example
10 : 1 or 1 : 4, in order to obtain by dilution in 0.1 M
sodium bicarbonate the overall final concentrations which
are indicated above but which in the case of mixtures of
several peptides contain the latter in equal
concentrations (in the case of 1 : 1 mixture) or in
various ratios to one another.

In each case, 100 μ l of each dilution were placed in 16
20 wells of microtiter plates, type B supplied by Nunc,
Roskilde, Denmark. The test plates charged with the
dilutions were left at 20°C for 18 hours and then the
solutions in the wells were removed by aspiration and the
wells were washed 3 - 4 times with 300 μ l of a solution
25 of 10 g/l bovine serum albumin in phosphate-buffered
physiological saline (PBS, pH 7.4) by filling and removal
by aspiration, and the test plates were subsequently
dried over silica gel at 20°C.

Example 2

30 Preparation of a peroxidase-labeled antibody against
human immunoglobulin of IgG class (h-IgG) and TMB
substrate for detection

Antibodies against h-IgG were raised by the method of

KOEHLER and MILSTEIN for preparing monoclonal antibodies (Nature 256, 495, 1975), different monoclonal antibodies with the same antigen specificity being identified by the method described by STÄHLI et al. (J. of Immunological Methods 32, 297 - 304, 1980). After purification by gel chromatography and dialysis against phosphate-buffered saline (PBS, pH 7.4), the pool containing the monoclonal antibody fraction (4 mg of protein/ml) was subsequently reacted with N-gamma-maleimidobutyryloxysuccinimide (GMBS), obtained from Behring Diagnostics, as described by TANAMORI et al. (J. Immunol. Meth. 62, 123 - 131, 1983).

2-Iminothiolane hydrochloride (supplied by Sigma, Cat. No. I 6256) was reacted with horseradish peroxidase (POD), obtained from Boehringer Mannheim, Cat. No. 413470, as described by KING et al. (Biochem. 17, 1499 - 1506, 1978). An IgG-POD conjugate was prepared from the GMBS-IgG conjugate and the iminothiolane-POD conjugate as described by TANAMORI et al. (supra).

The resulting solution of the IgG-POD conjugate had a protein content of 360 µg/ml. The ratio of POD to IgG was determined as 2.8. The solution was subsequently diluted to 500 ng/ml IgG-POD with a solution of 50 ml/l fetal calf serum (FCS), 5 g/l polyoxyethylene(20)sorbitan monolaurate (^RTween 20) in PBS and was called anti-IgG/POD conjugate. For use in the ELISA, the subsequent dilution in tris buffer pH 7.4, containing 0.5% ^RTween 20, was varied (between 1 : 100 and 1 : 20,000 dilution) in order to prepare uniformly a 1 : 26 final dilution in conjugate buffer, containing 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), 0.1 M sodium chloride (NaCl) and 0.1% ^RTween pH 8.4. Rabbit polyclonal antibodies prepared according to the state of the art were adjusted so that a dilution of 1 + 25 was likewise achieved for use.

Used for the detection of anti-IgG/POD conjugate was a

substrate system or a substrate preparation containing hydrogen peroxide and tetramethylbenzidine (TMB), which was prepared from two stock solutions.

5 Stock solution 1: TMB dihydrochloride was dissolved while stirring in a concentration of 5 g/l, i.e. of 16 mmol/l, in double-distilled water and adjusted to pH 1.5 with 5 normal hydrochloric acid. Penicillin G was added to this solution while stirring in a final concentration of 200 mg/l, i.e. of 0.56 mmol/l.

10 Stock solution 2: 1.4 ml of glacial acetic acid, 1.5 ml of 1 normal NaOH and 250 mg, i.e. 3 mmol, of H₂O₂ as urea/hydrogen peroxide adduct were added to 900 ml of double-distilled water. After dissolution was complete, the mixture was made up to 1 l with double-distilled water.

15 TMB substrate preparation: One part by volume of stock solution 1 and 10 parts by volume of stock solution 2 were mixed together.

Example 3

State of the art

20 Employed as example was a commercially available ELISA test kit (HP1) in which the C-100-3 construct which is described in WO 89/04669 and is prepared in yeast by genetic engineering is used as antigen. The procedure for investigating human sera and plasmas was that indicated in the manufacturer's pack insert, for example 1 : 11 sample dilution and 1 h incubation of serum, 1 h incubation of anti-human IgG/POD conjugate and the enzyme substrate system with o-phenylenediamine (OPD) as substrate, photometric measurement at 492 nm and establish-
25 ment of limits (a threshold of 0.40 E is added to the mean of the negative controls).

30

An entirely analogous procedure was used with another

commercially available ELISA (HP 2) which, besides the C-100 construct, contains additional epitopes from the core region and from the NSP 3 region (c33c), that is to say the original test kit was used, observing all the statements about procedure stated by the manufacturer - as described above, for investigating human samples.

By contrast, the procedure for determining HCV-specific antibodies in chimpanzee serum or plasma with the two commercial products was such that a polyclonal antibody raised in rabbits against human IgG was used for detection. For this purpose, the IgG fraction of rabbit antiserum was, as described in Example 2, purified, dialyzed and labeled with peroxidase (POD). The final concentration was adjusted to about 4 times the concentration of the monoclonal anti-IgG/POD conjugate in order reliably to fashion over the cross-reaction, validated in preliminary tests, of the antibodies against human IgG for chimpanzee IgG.

It was necessary to modify the establishment of limits as described in Tab. 10 A - C because the initial values of all the chimpanzees already exhibited elevated values (see Tab. 10 A - C).

The comparative anti-HIV 1 + 2 combination test for the non-differentiating determination of HIV 1 and HIV 2 antibodies is a commercially available product (HP 3) which is based on synthetically prepared peptides of HIV 1 and HIV 2. The procedure for this test was also that of the manufacturer's pack insert, for example sample dilution 1 : 2, 30 min incubation of serum, 30 min incubation of conjugate (anti-human IgG/POD) and the enzyme substrate system with tetramethylbenzidine (TMB) as substrate, photometric measurement at 450 nm and establishment of limits (a threshold of 0.250 was added to the mean of the negative controls).

Example 4

Determination of human antibodies of immunoglobulin class G against HCV in the ELISA with the peptides according to the invention

5 50 μ l of serum or plasma were added to 50 μ l of sample
buffer containing 0.3 M tris, 0.3 M NaCl, 20% boviserine
and 0.1% RTween 20 in wells of microtiter plates which
were coated as described with peptides or peptide mix-
tures. After incubation at 37°C for 30 min, the content
10 of the wells was removed by aspiration, and the wells
were washed five times with washing buffer containing 1
g/l RTween 20 in PBS. Then 100 μ l of conjugate in the
final dilution were added to the wells, preferably using
a preliminary dilution of 1 : 3000 in tris, 0.5% Tween 20
and a final dilution of 1 : 26 in conjugate buffer. After
incubation at 37°C for 30 min, the content of the wells
was removed by aspiration and again washed five times.
Subsequently 100 μ l of TMB substrate preparation were
added to each well, incubated at 20 - 22°C for 30 min,
and incubation was stopped by addition of 100 μ l of 1
normal sulfuric acid. The extinction of the colored
solution was measured at a wavelength of 450 nm (E_{450})
with a PBS blank as reference.

25 Samples classified as anti-HCV positive were those which
produced an E_{450} greater than 0.10, samples classified as
anti-HCV marginal were those whose E_{450} was in the range
from 0.05 to 0.10, and samples classified as anti-HCV
negative were those which produced an E_{450} below 0.05.

30 Tab. 1 summarizes results obtained from the determina-
tion, described in Example 1, 2 and 4, of human samples
with the peptide 4083 according to the invention and with
a mixture of smaller sequences of the formula I. The data
in Tab. 2 were obtained analogously with the peptide SP
35 10 according to the invention, the results of both ELISAs
being compared with those of HP 1 (Example 3).

Results of the determination of anti-HCV with an ELISA containing the peptide 4083 and with a mixture of smaller peptides on the solid phase with human samples

Positive with peptide ELISA				
(2 µg 4083/ml)			(2 µg 4060/ml and 2 µg 4082/ml)	
initial positive		retest positive	retest positive	
anti-HCV				
positive samples				
from France	n=15	15	15	15
from Austria	n=17	17	17	17
from Germany	n=20	20	20	20
from USA	n=49	49	49	49
total	n=101	101 ¹⁾	101 ¹⁾	101 ²⁾
paired sera/plasma from healthy blood donors				
n = 259 sera		2	1	n.d.
n = 259 plasmas		1	0	n.d.

1) n = 97 samples showed extinctions > 2.5 E which resulted in ratios > 25 at a limit of 0.10 E

2) n = 94 samples showed extinctions > 2.5 E.

Only 4 and 7 samples, respectively, reacted more weakly, but with E450 values between 0.8 and 2.5 and a limit of 0.10 E still rather strongly.

Tab. 2

Results of the determination of anti-HCV with an ELISA containing the novel peptide SP 10 on the solid phase with human samples

		Positive with peptide ELISA (2 µg SP 10 /ml)	
Anti-HCV positive samples		initial positive	retest positive
10	from France n = 35	35	35
	from Austria n = 26	26	26
	from Germany n = 29	29	29
	from USA n = 53	53	53
15	total	143	143 ¹⁾
Paired sera/plasmas (see distribution plot of healthy blood donors in Fig. 2)			
	n = 500 sera	0	0
20	n = 500 plasmas	0	0

1) n = 142 samples showed extinctions > 2.5 E, which results in ratios >> 25 at a limit of 0.1 E; only one sample reacted more weakly with a value of 1.2 E, but still considerably more strongly than with the commercial ELISA (HP 1).

All of the tested human samples which were classified as anti-HCV positive with a commercial test (HP1) were likewise found to be positive with all ELISAs based on the peptides according to the invention. Besides the very good agreement with the results of the commercial test (HP1), the very strong signal formation in the peptide ELISAs is also noticeable. At the same time, the results of the longitudinal investigation of healthy blood donors makes it clear that the susceptibility of the test to interference is extremely low. Thus, on testing of

healthy donors of serum and plasma there was found to be only extremely minimal non-specific binding to the peptides according to the invention, i.e. only a few false positive results were obtained.

5 Tab. 3 presents further results obtained on testing of human samples with ELISAs based on the use of smaller sequences (15 amino acids) of the formula (I) according to Examples 1,2 and 4. It is clear that the peptides are
10 suitable for epitope definition of HCV antibodies and as mixture of several peptides, preferably containing 3 to 6 peptides, for the determination of anti-HCV.

Tab. 3

Reactivities of human anti-HCV-positive samples in ELISAs with smaller peptides comprising 15 amino acids on the surface of microtiter plates according to Example 1; the results are reported as extinctions at 450 nm (E_{450}) (- = negative finding below 0.10 E).

	4056	4055	4053	4052	4081	4091	Mixture
Proben-							4056
Nr.							4055
							4053
							4052
							4081
							4091
$\mu\text{g/ml}$	2	2	2	2	2	2	0.5 each
BC90-89	>2.50	0.20	-	-	-	-	1.40
252	-	-	0.20	-	>2.50	-	2.10
90	0.80	-	-	-	1.60	-	2.00
268	>2.50	-	0.20	-	>2.50	>2.50	>2.50
84	-	1.20	1.60	-	1.70	>2.50	>2.50
325	>2.50	>2.50	-	-	>2.50	>2.50	>2.50
270	2.00	1.70	-	-	2.20	>2.50	>2.50
229	0.20	-	-	1.10	1.00	-	1.50
288	>2.50	>2.50	-	0.50	>2.50	>2.50	>2.50
290	>2.50	>2.50	-	0.20	>2.50	>2.50	>2.50
242	-	0.50	-	-	1.20	1.40	>2.50
137	-	-	-	-	0.40	0.35	0.70
286	-	-	0.60	0.15	0.45	0.15	0.90
192	0.20	>2.50	-	1.00	-	-	>2.50
235	0.15	-	0.40	-	0.20	-	0.30
85	-	-	-	0.75	-	-	0.40

The reactivities obtained with a mixture of peptides according to the invention are shown. It is evident that, owing to the very strong signal formation of the peptide ELISA, a reliable discrimination between positive and negative results is achieved. Analogous results were also achieved with the following peptides or peptide mixtures according to the invention.

4074/4081 (2 $\mu\text{g/ml}$ each)

4074/4082 (0.5 and 0.125 $\mu\text{g/ml}$)

4060/4071/4081 (2 $\mu\text{g/ml}$ each), where mixtures of smaller peptides comprising about 15 AA proved thoroughly suitable:

4056/4055 and 4052 (0.5 $\mu\text{g/ml}$ each).

No dependence of the reactivity of the samples on their geographical origin was detectable in any case on testing with all the peptides according to the invention.

Example 5

Optimization of the ELISA determination

The modifiable parameters of the ELISA determination which were varied were mainly the peptide concentration used for the coating, or in the case of mixtures of peptides their total concentration and ratio to one another, at a constant conjugate concentration of 1 : 3000 and 1 : 26 dilution. In addition, the conjugate preliminary dilution was varied at fixed coating concentrations. Both modifiable variables were validated with regard to specificity by testing blood donors' sera and plasmas and with regard to sensitivity by determination of anti-HCV in positive groups. Furthermore, the limiting sensitivity was measured in the form of the analytical sensitivity by serial dilution of anti-HCV-positive samples (1 : 2, 1 : 4 etc. in anti-HCV-negative sera) and compared with the data of a commercial test in just the same way as with the results achieved using the peptide described in the literature.

The results obtained with human samples are compiled in Tab. 4 by way of example.

Tab. 4

Comparative titration of anti-HCV-positive sera and plasmas in the peptide ELISA (4083) and a commercially available test. The ratios are reported as quotients of the specific signals to the limit, and values greater than 1 indicate a positive result and values smaller than 1 indicate a negative result.

Serum Dilution in neg. serum		Peptide ELISA (4083) 2 µg/ml 4083 limit 0.10 E	Commercial test (HP1) limit 0.454 E
HC 90-84	1: 1	> 25	> 6
	1: 2	> 25	> 6
	1: 4	> 25	> 6
	1: 8	> 25	> 6
	1: 16	24	> 6
	1: 32	10.7	4.1
	1: 64	6.1	2.0
	1: 128	2.5	<u>0.8</u> neg.
	1: 256	1.5	0.5
	1: 512	1.1	0.5
	1:1024	<u>0.6</u> neg.	0.2
HC 90-90	1: 1	> 25	> 6
	1: 2	> 25	> 6
	1: 4	> 25	> 6
	1: 8	> 25	> 6
	1: 16	> 25	> 6
	1: 32	11.8	5.4
	1: 64	5.9	3.7
	1: 128	3.3	1.4
	1: 256	1.8	<u>0.9</u> neg.
	1: 512	1.08	0.4
	1:1024	<u>0.5</u> neg.	0.3
	1:2048	0.4	0.2
HC 90-252	1: 1	> 25	> 6
	1: 2	> 25	> 6
	1: 4	> 25	4.5
	1: 8	20	3.2
	1: 16	12.2	2.7
	1: 32	6.5	1.2
	1: 64	4.2	<u>0.8</u> neg.
	1: 128	2.4	0.5
	1: 256	<u>0.97</u> marg. ¹⁾	

¹⁾ marg. = marginal

Continuation of Tab. 4

Serum Dilution in neg. plasma	Peptide ELISA (4083) 2 µg/ml 4083 limit 0.10 E		Commercial test (HP1) limit 0.454 E
HC 90-296	1: 1	> 25	> 6
	1: 2	> 25	> 6
	1: 4	20	> 6
	1: 8	8,5	5,0
	1: 16	3,7	2,9
	1: 32	2,9	<u>0,7</u> neg.
	1: 64	1,4	0,5
	1:128	<u>0,8</u> neg.	0,4
	1:256	0,3	0,2
HC 90-83	1: 1	> 25	> 6
	1: 10	20	2,1
	1:100	6,5	<u>0,4</u> neg.
HC 90-240	1: 1	> 25	> 6
	1: 10	8	1,5
	1:100	1,4	<u>0,3</u> neg.
HC 90-239	1: 1	> 25	> 6
	1: 10	7,8	2,0
	1:100	1,4	<u>0,1</u> neg.
HC 90-89	1: 1	> 25	> 6
	1: 10	> 25	> 6
	1:100	9	3,5
HC 90-137	1: 1	> 25	> 6
	1: 1	21	4,2
	1:100	4	1,4

The results in Tab. 4 make it clear that the sensitivity of the ELISA based on the peptides according to the invention, measured by the limiting sensitivity of serum titrations, is at least as good as the commercial test (HP1) which was tested comparatively with identical serum dilutions. In fact, in many cases, diluted samples which already gave repeated negative reactions in the

commercial test (HP1) were still measured as significantly positive with the peptide ELISA so that the overall result was a better detection limit of HCV antibodies with the peptides according to the invention. Analogous results were likewise achieved with other peptide concentrations, peptide sequences or mixtures of the novel peptides; such as, for example,

4083 (0.25 $\mu\text{g/ml}$)
4074/4081 (0.25 $\mu\text{g/ml}$ each)
4074/4082 (0.5 $\mu\text{g/ml}$ each)
4074/4082 (0.5 and 0.125 $\mu\text{g/ml}$)
4060/4082 (2 $\mu\text{g/ml}$ each)

Beyond the determination of the limiting sensitivity, in the case of optimized systems the specificity was also examined by means of non-HCV-specific antibodies and other potential interfering factors, and it became clear that the anti-HCV determination with the peptides according to the invention is specific for HCV and is not subject to any known interferences such as, for example, cross-reactions of other antibodies, interference by heat inactivation or the like. This also applies correspondingly to other stated novel peptides or peptide mixtures, with which uniformly a high serum concentration (dilution 1 : 2 in sample buffer) was used in order to increase the sensitivity, which was possible owing to the purity of the peptides and the high antigen density on the solid phase.

The comparisons summarized in Tab. 5 between another core peptide according to the invention, SP10, and HP1 likewise make it clear that the peptide according to the invention has, compared with the state of the art, advantages which in turn comprise improved limiting sensitivities, i.e. increased analytical sensitivity. Thus, the 4 titrations in Tab. 5 were determined a factor of 2 to 4 more sensitively than with the published peptide, and the factor was even 8 to 32 compared with a commercial test (HP1).

11 91 87002

Tab. 5

Comparison of the analytical sensitivity of an ELISA based on a core peptide (Sp10, 2 $\mu\text{g/ml}$) with the state of the art. For this, serial dilutions of anti-HCV-positive human sera in negative human serum were prepared and the results of the tests with an ELISA based on a peptide according to the invention (SP10) were compared with the commercial HCV ELISA (HP1) and with a peptide described as immunorelevant according to Fig. 2 in the literature (OKAMOTO et al.). All the data represent absorbances ($E_{450 \text{ nm}}$), the results to be regarded as reactive being underlined.

Serum No.	Dilution	Peptide SP10 according to the invention		Peptide disclosed in the literature (OKAMOTO)		Commercial anti-HCV ELISA (HP1)
		AA 1 - 30 2 $\mu\text{g/ml}$	0.25 $\mu\text{g/ml}$	AA 39 - 74 2 $\mu\text{g/ml}$	0.25 $\mu\text{g/ml}$	
HC 90-85	1:1	> 2,500	> 2,500	> 2,500	> 2,500	> 2,500
	1 : 32	> 2,500	> 2,500	1,008	0,878	<u>0,908</u>
	1 : 64	1,586	0,742	0,548	0,477	0,342 -
	1 : 128	1,032	0,783	0,250	<u>0,183</u>	0,153 -
	1 : 256	0,601	0,519	<u>0,101</u>	0,098	
	1 : 512	0,328	0,232	0,060	0,051	
	1 : 1024	<u>0,188</u>	<u>0,122</u>			
	1 : 2048	0,080	0,034			
HC 90-225	1:1	> 2,500	> 2,500	> 2,500	> 2,500	> 2,500
	1 : 16	> 2,500	> 2,500	> 2,500	2,366	> 2,500

1191

87002

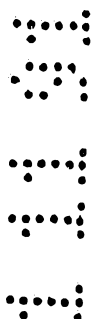
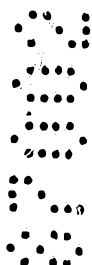
Continuation of Tab. 5

1 : 32	> 2,500	2,065	2,020	1,619	> 2,500	
1 : 64	1,669	1,520	0,655	0,883	1,759	
1 : 128	0,971	0,790	0,414	0,544	<u>0,909</u>	
1 : 256	0,561	0,374	0,281	0,215	0,380	-
1 : 512	0,486	0,191	<u>0,146</u>	<u>0,157</u>	0,122	-
1 : 1024	<u>0,295</u>	<u>0,141</u>	0,080	0,115		
HC 90-270 1:1	> 2,500	> 2,500	> 2,500	> 2,500	> 2,500	
1 : 8	> 2,500	> 2,500	> 2,500	2,222	1,902	
1 : 16	> 2,500	2,500	1,478	1,016	0,980	
1 : 32	1,512	1,408	0,743	0,478	<u>0,501</u>	
1 : 64	0,898	0,802	0,322	<u>0,216</u>	0,259	-
1 : 128	0,536	0,483	<u>0,121</u>	0,096	0,179	-
1 : 256	<u>0,312</u>	<u>0,275</u>	0,050	0,041		
HC 90-354 1: 1	> 2,500	> 2,500	> 2,500	> 2,500	> 2,500	
1 : 32	> 2,500	> 2,500	1,917	0,786	<u>0,496</u>	+/-
1 : 64	> 2,500	1,833	1,282	0,527	0,352	-
1 : 128	1,703	0,873	0,685	<u>0,249</u>	0,200	-
1 : 256	0,918	0,410	0,367	0,082		
1 : 512	0,582	0,310	<u>0,145</u>			
1 : 1024	0,326	<u>0,178</u>	0,072			
1 : 2048	<u>0,159</u>	0,084				

141

5 Evaluation of another core peptide according to the invention (SP 23) gave similar results. The comparative results presented in Tab. 6 reveal that SP 23 is equivalent to SP 10 with respect to sensitivity, and both peptides are superior to the peptide described in the literature.

10 Although the literature-analogous peptide SP 12 (AA 47-75) does not correspond exactly to the peptide described by OKAMOTO et al. (AA 39-74), the results on the intermediate sequence (SP 11, AA 24-53, Tab. 6) make it clear that no immunorelevant epitopes are present in the following sequence region (AA 39 - 47), and the very weak reactivity of SP 11 which is still detectable is attributable to the carboxyl-terminal region of SP 10 contained
15 therein (overlapping region AA 24-30 shown in Fig. 3).



Tab. 6:

Comparison of the immunoreactivities in the ELISA between 2 peptides according to the invention and an amino-acid sequence which is located between the published sequence (AA 39-74, OKAMOTO et al.) and the novel structures (SP 11, AA 24 - 53, see also Fig. 3). All data represent extinctions ($E_{450 \text{ nm}}$).

Samples	SP 23	SP 10	SP 11
	AA 8-26	AA 1-30	AA 24-53
	2 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$
pos. contr.	> 2.500	> 2.500	0.405
neg. contr.	0.051	0.049	0.030
Dilutions			
HC-90-225			
1 : 16	2.203	> 2.500	0.428
1 : 64	1.329	1.367	<u>0.164</u>
1 : 256	0.454	0.386	0.024
1 : 1024	<u>0.195</u>	<u>0.142</u>	
HC-90-354			
1 : 16	> 2.500	> 2.500	<u>0.164</u>
1 : 64	1.413	2.156	0.059
1 : 256	0.418	0.496	
1 : 1024	<u>0.124</u>	<u>0.162</u>	
Anti-HCV-positive samples			
(commercial test HP 1)			
HC 90-371	<u>2.200</u>	<u>2.086</u>	<u>0.224</u>
HC 90-336	> <u>2.500</u>	> <u>2.500</u>	<u>0.335</u>
HC 90-453	> <u>2.500</u>	> <u>2.500</u>	0.058
HC 90-570	<u>1.877</u>	<u>2.277</u>	0.088

This surprising advantage in sensitivity becomes particularly clear on testing undiluted samples. Thus, all 11

of the 11 anti-HCV-positive samples shown in Tab. 7 were found to be reactive with the peptide according to the invention, whereas the peptide described in the literature finds only 6 samples positive, and in no case was there a positive reaction with the commercial test (HP1). With respect to the samples which agree in reacting positively with both peptides, it is noticeable that the peptide according to the invention reacts significantly more strongly under identical test conditions, which provides considerable advantages especially for positive/negative discrimination.



111 31 87002

Tab. 7

Comparison of the sensitivity of a peptide ELISA (SP10 2 μ g/ml) with the state of the art. For this, 11 selected native human anti-HCV-positive samples were tested with an ELISA based on a peptide according to the invention, comparing with a commercial test (HP1) and with a peptide described as immunorelevant according to Fig. 3 in the literature (OKAMOTO et al.). All the data represent extinctions ($E_{450 \text{ nm}}$). The anti-HCV-positive sample HC 90-495 was also tested as control.

Serum No.	Peptide according to the invention SP10 2 μ g/ml cut off: 0.100 E	Peptide described in the literature (OKAMOTO) SP12 2 μ g/ml cut off: 0.100 E	Commercial HCV ELISA (HP1) cut off 0.454 E	
HC 90-224	1,952	2,005	0,114	Neg.
HC 90-284	2,417	0,451	0,095	Neg.
HC 90-289	> 2,500	1,220	0,278	Neg.
HC 90-300	> 2,500	0,288 marg.	0,208	Neg.
HC 90-323	> 2,500	> 2,500	0,313	Neg.
HC 90-493	> 2,500	0,051 Neg.	0,088	Neg.
HC 90-509	> 2,500	0,081 Neg.	0,083	Neg.
HC 90-512	> 2,500	0,049 Neg.	0,110	Neg.
HC 90-531	2,208	0,257 marg.	0,081	Neg.
HC 90-536	2,500	0,234 marg.	0,069	Neg.
HC 90-494	1,279	n.d.	0,114	Neg.
HC 90-495	> 2,500	> 2,500	> 2,500	Pos.

These findings (Tab. 7) derived from the titrations (Tab. 5) and preselected native human sera with the core peptides are confirmed by the results of the tests on commercially available serum panel (LOT # PHV 101 and LOT # PHV 201, Boston Biomedica, USA).

Specifically, the results of the so-called low-titer anti-HCV panel are summarized in Tab. 8 and the results of the mixed-titer anti-HCV panel are summarized in Tab. 9. The results of the data obtained with the core peptide according to the invention are compared to the data obtained with commercial tests as state of the art.

Tab. 8

Tab. 8 shows the comparison of the sensitivity of a peptide ELISA (SP 10, 2 μ g/ml) with the state of the art for native anti-HCV-positive samples in the panel PHV 101, comprising 15 low-titer well-characterized anti-HCV-positive samples (material and comparative test results marketed by Boston Biomedica, USA). All ELISA data are ratio values, which describes the ratio of sample extinction to cut-off. Values < 1.0 are regarded as negative and > 1.0 are regarded as positive.

1191 87002

Tab. 8

Member I.D. <u>Number</u>	Comparative results		<u>RESULT</u>	ELISA with peptide according to the invention (SP10/2 µg/ml)
	HCV EIA <u>S/CO</u>	HCV ELISA <u>S/CO</u>		<u>S/CO</u>
PHV101-01	3.717	0.964	POS	18 +
PHV101-02	0.167	0.268	N	0.25 -
PHV101-03	1.880	1.323	POS	0.90 -
PHV101-04	1.757	3.076	POS	> 25
PHV101-05	3.404	2.169	POS	4.0
PHV101-06	1.640	1.642	POS	> 25
PHV101-07	2.281	1.624	POS	> 25
PHV101-08	3.009	1.588	POS	> 25
PHV101-09	1.855	1.119	POS	> 25
PHV101-10	3.649	3.098	POS	> 25
PHV101-11	0.662	1.820	POS	> 25
PHV101-12	3.018	2.205	POS	> 25
PHV101-13	2.805	2.770	POS	> 25
PHV101-14	1.912	0.846	POS	> 25
PHV101-15	3.686	3.240	POS	> 25

11191

87002

Continuation of Tab. 8

MEMBER I.D. Number	CONFIRMATORY DATA RIBA 2.0				RESULT*	ALT**	anti-HBc*	HBsAg*	anti-HIV*	anti-HTLV*
	5-1-1	C-100-3	C33C	C22-C						
PHV101-01	1	+/-	+/-	2	POS	L	N	N	N	N
PHV101-02	-	-	-	-	N	L	N	N	N	N
PHV101-03	+/-	+/-	+/-	-	N	L	N	N	N	N
PHV101-04	+/-	+/-	4	4	POS	L	POS	N	N	N
PHV101-05	-	+/-	-	+/-	N	L	N	N	N	N
PHV101-06	+/-	-	1	4	POS	L	POS	N	N	N
PHV101-07	-	+/-	1	4	POS	L	POS	N	N	N
PHV101-08	+/-	-	1	4	POS	G	POS	N	N	N
PHV101-09	-	-	1	4	POS	L	N	N	N	N
PHV101-10	1	-	2	4	POS	L	POS	N	N	N
PHV101-11	1	+/-	1	1	POS	L	N	N	N	N
PHV101-12	2	-	4	4	POS	L	POS	N	N	N
PHV101-13	+/-	+/-	4	3	POS	L	POS	N	N	N
PHV101-14	+/-	-	1	3	POS	L	POS	N	N	N
PHV101-15	1	-	3	4	POS	L	POS	N	N	N

*POS = Positive, N = Negative, NA = Not applicable

**L = Less than 2 x upper limit of normal, G = Greater than 2 x upper limit of normal.

ALT results are those read following sterile filtration and vialing and may not reflect the ALT status of the donor at the time of donation.

It is evident from the data in Tab. 8 that, with one exception (PHV 01-03), all anti-HCV-positive samples give a correctly positive reaction with the novel peptide. It is noticeable, by comparison with the commercial test, that the signal strength, expressed by the ratio sample signal: cut off, was found to be significantly stronger with the peptide according to the invention than with the assays tested for comparison, which expresses a distinctly improved reliability of the novel peptide ELISA.

According to these data (ratio > 25, which corresponds to extinctions > 2.5), the samples are low-titer only according to the definition of the state of the art and are found to be, surprisingly, strongly reactive with the novel peptide.

The one exception (sample 03) is explained by the data of the comparative results on the confirmatory test which demonstrates absence of core-specific antibodies in this sample (Q22c is the core-protein prepared in E. coli by genetic engineering). According to this test, sample 03 ought in fact to be classified as negative.

The tests on the second panel summarized in Tab. 9 led to similar observations: 19 of the total of 22 anti-HCV-positive samples are found, surprisingly, to be extremely strongly positive with extinctions greater than 2.5 (corresponds to ratio > 25). The three anti-HCV-negative sera contained in the panel are correctly classified as negative (ratios < 1.0). Finally, samples Nos. PHV 201-08, -10 and -20 are also found to be correct within the scope of the problem because these samples exhibit no (-08 and -10) or few (-20) core-specific antibodies according to the confirmatory test.

Tab. 9 shows the comparison of the sensitivity of a peptide ELISA (SP 10, 2 µg/ml) with the state of the art for native anti-HCV-positive samples in the panel

- 5 PHV 201, comprising well-characterized anti-HCV-positive samples with different anti-HCV titers. (Material and comparative test results marketed by Boston Biomedica, USA). All the ELISA data are given as ratios which represent the quotients of sample extinction and cut-off value. Values < 1.0 represent a negative result and values > 1.0 represent a positive result.

1 1 91

07002

Tab. 9

Comparative results

MEMBER I.D. NUMBER	HCV EIA	HCV ELISA	RESULT*	ELISA with peptide according to the invention (SP 10/ 2 µg/ml)
				S/Co
MIV201-01	1.609	1.475	POS	> 25
MIV201-02	3.660	5.667	POS	> 25
MIV201-03	3.793	3.946	POS	> 25
MIV201-04	0.271	0.176	N	0.10 -
MIV201-05	3.417	2.591	POS	> 25
MIV201-06	3.711	4.005	POS	> 25
MIV201-07	0.260	0.108	N	0.25 -
MIV201-08	1.515	1.244	POS	0.75 -
MIV201-09	2.530	1.274	POS	> 25
MIV201-10	2.174	2.745	POS	0.25 -
MIV201-11	2.003	2.169	POS	> 25
MIV201-12	3.641	4.769	POS	> 25
MIV201-13	4.115	5.662	POS	> 25
MIV201-14	2.669	4.300	POS	> 25
MIV201-15	4.115	5.660	POS	> 25
MIV201-16	4.115	5.662	POS	> 25
MIV201-17	4.115	5.662	POS	> 25
MIV201-18	3.530	3.943	POS	> 25
MIV201-19	0.490	1.520	N	0.25 -
MIV201-20	2.611	2.377	POS	> 25
MIV201-21	3.430	2.666	POS	> 25
MIV201-22	1.721	1.690	POS	> 25
MIV201-23	4.115	5.662	POS	> 25
MIV201-24	2.500	2.631	POS	> 25
MIV201-25	4.115	5.660	POS	> 25

MEMBER CONFIRMATORY DATA

I.D. RIBA 2.0

ELISA with peptide
according to the invention
(SP 10/ 2 µg/ml)

NUMBER	5-1-1	C-100-3	C33C	C22-C	RESULT*	ALT**	anti-HBc*	HBsAg*	anti-HIV*	anti-HIV*	S/Co
PIV201-01	+/-	-	2	4	POS	L	N	N	N	N	> 25
PIV201-02	1	2	4	4	POS	L	POS	N	N	N	> 25
PIV201-03	-	1	-	4	POS	L	N	N	N	N	> 25
PIV201-04	-	-	-	-	N	L	N	N	N	N	0.10 -
PIV201-05	2	+/-	+/-	4	POS	L	N	N	N	N	> 25
PIV201-06	2	1	4	4	POS	L	N	N	N	N	> 25
PIV201-07	-	-	-	-	N	L	N	N	N	N	0.25 -
PIV201-08	-	+/-	3	-	I	L	POS	N	N	N	0.75 -
PIV201-09	1	-	4	4	POS	L	POS	N	N	N	> 25
PIV201-10	+/-	+/-	2	-	I	G	N	N	N	N	0.25 -
PIV201-11	2	+/-	3	3	POS	G	N	N	N	N	> 25
PIV201-12	2	2	2	3	POS	L	N	N	N	N	> 25
PIV201-13	4	4	4	4	POS	L	N	N	N	N	> 25
PIV201-14	2	+/-	4	4	POS	L	POS	N	N	POS	> 25
PIV201-15	2	+/-	4	4	POS	L	N	N	N	N	> 25
PIV201-16	4	4	4	4	POS	L	N	N	N	N	> 25
PIV201-17	4	4	4	4	POS	L	POS	N	N	N	> 25
PIV201-18	+/-	2	4	3	POS	L	POS	N	N	N	> 25
PIV201-19	-	+/-	-	-	N	L	N	N	N	N	0.25 -
PIV201-20	1	1	1	+/-	POS	L	N	N	N	N	> 25
PIV201-21	1	+/-	3	4	POS	L	POS	N	N	N	> 25
PIV201-22	+/-	-	4	4	POS	L	N	N	N	N	> 25
PIV201-23	4	3	4	4	POS	L	POS	N	N	N	> 25
PIV201-24	2	4	4	4	POS	G	POS	POS	N	N	> 25
PIV201-25	4	1	4	4	POS	L	N	N	N	N	> 25

*POS = Positive, N = Negative, I = Indeterminant, NA = Not applicable

**L = Less than 2 x upper limit of normal, G = Greater than 2 x upper limit of normal.

ALT results are those read following sterile filtration and vialing and may not reflect the ALT status of the donor at the time of donation.

Example 6

Determination of anti-HCV in chimpanzee sera with the peptides according to the invention in the ELISA

5 Various peptides and peptide mixtures adsorbed in wells
of microtiter plates were investigated with sera from
chimpanzees which had been infected with various infec-
tious doses of NANBV. The sequential samples taken every
2 weeks after inoculation underwent measurement of not
only GOT but also GPT with commercial tests, and all
10 samples were determined in parallel and in the ELISA with
peptides according to the invention. Similar to the
commercial test (HP1) in Example 3, in the peptide ELISA
the polyclonal rabbit antibodies, labeled with POD, were
used concentrated 4-fold for the detection, using the
15 enzyme substrate TMB with photometric measurement at
450 nm. The test procedure corresponded to the determina-
tion of human anti-HCV described above, with 0.1 E as
fixed limit and presentation of the results of Tab. 10 as
ratio of the specific extinctions to this cut-off value
20 (ratios).

The results listed in Tab. 10A to 10C and 11A and 11B with
peptides from the NSP 4 region represent so-called ratios
with which the quotient of specific signal cut off is
defined.

25 Tab. 10 A - C

Anti-HCV determination in chimpanzee samples with a
commercial test (HP1) and ELISAs with peptides or peptide
mixtures according to the invention from the NSP 4
region. The data for the first 3 time values are extinc-
tions which serve to fix the limits, from which the
30 subsequent ratios of specific signal and limit have been
formed.

11191

87002

Tab. 10 A

Animal No. 123*

	ALT	Commercial test (HP1)	4083 2 µg/ml	4083 0.5 µg/ml	4060 4081 2 µg/ml each
0 = inoculation	25	0,500 E	0,032 E	0,041 E	0,026 E
2	23	0,600 E ⁴⁹²	0,027 E ⁴⁵⁰	0,024 E ⁴⁵⁰	0,014 E ⁴⁵⁰
4	23	0,450 E	0,013 E	0,025 E	0,019 E
Extinctions (E) define the limits:		0,7 E	0,10 E	0,10 E	0,10 E
<u>ratios (r):</u>					
6	29	r 0,6	r 0,01	r 0,03	r 0,03
8	30	r 0,4	r 0,02	r 0,03	r 0,03
10	50	r 0,7	r 0,01	r 0,02	r 0,02
12	55	r 0,4	r 0,02	r 0,03	r 0,03
14	50	r 0,5	r 0,01	r 0,03	r 0,03
16	140	r 0,6	r 0,03	r 0,03	r 0,03
18	> 170	r 0,7	r 5,3 <u>pos.</u>	r 1,5 <u>pos.</u>	r 0,6
20	30	r 0,6	r 7,4 <u>pos.</u>	r 1,8 <u>pos.</u>	r 0,7
22	23	r 0,5	r 5,7 <u>pos.</u>	r 1,1 <u>pos.</u>	r 0,5
24	23	r 0,6	r 2,7 <u>pos.</u>	r 0,7	r 0,3
26	30	r 0,6	r 1,5 <u>pos.</u>	r 0,4	r 0,3

*) confirmed as NANB-infected by electron microscopic investigation of liver biopsies

11 91 87002

Tab. 10 B

Animal No. 048		ALT	Commercial test (HP1)	4083	4083	4060	4073	4090
Time (weeks)				2 µg/ml	0.5 µg/ml	4081 2µg/ml each	2 µg/ml	2 µg/ml
0 = inoculation	20	0,5 E ⁴⁹²	0,018 E ⁴⁵⁰	0,027 E ⁴⁵⁰	0,008 E ⁴⁵⁰	0,036 E ⁴⁵⁰	0,028 E ⁴⁵⁰	
2	25	0,5 E	0,041 E	0,070 E	0,007 E	0,041 E	0,050 E	
4	23	0,5 E	0,026 E	0,050 E	0,009 E	0,039 E	0,037 E	
Extinctions (E) define the limits:		0,70 E	0,10 E	0,10 E	0,10 E	0,10 E	0,10 E	
<u>ratios (r)</u>								
10	32	r 0,6	r 0,3	r 0,5	r 0,2	r 0,6	r 0,6	
12	100	r 0,4	r 1,0 marg. ¹⁾	r 1,0 marg. ¹⁾	r 0,1	r 0,95 marg. ¹⁾	r 2,0 pos.	
14	112	r 0,7	r 11,7 pos.	r 4,9 pos.	r 0,1	r 6,9 pos.	r 16,2 pos.	
16	30	r 1,0	r 7,4 pos.	r 3,8 pos.	r 0,2	r 12,2 pos.	r 23,2 pos.	
18	25	r 0,7	r 7,2 pos.	r 2,8 pos.	r 0,2	r 7,8 pos.	r 18,9 pos.	
20	30	r 0,8	r 8,6 pos.	r 4,7 pos.	r 0,1	r 12,8 pos.	r 16,4 pos.	
22	25	r 0,8	r 8,4 pos.	r 5,4 pos.	r 0,1	r 11,7 pos.	r 22,3 pos.	
24	23	r 0,8	r 9,8 pos.	r 7,2 pos.	r 0,2	r 15,5 pos.	r 20,0 pos.	
26	20	r 0,8	r 10,8 pos.	r 6,0 pos.	r 0,2	r 13,0 pos.	r 17,4 pos.	
28	20	r 0,8	r 9,8 pos.	r 5,4 pos.	r 0,2	r 11,7 pos.	r 8,9 pos.	
30	20	r 1,0 marg. ¹⁾	r 7,6 pos.	r 6,3 pos.	r 0,2	r 11,5 pos.	r 12,9 pos.	
32	25	r 1,3 pos.	r 16,2 pos.	r 17,4 pos.	r 1,3 pos.	r 24,5 pos.	r 23,3 pos.	
34	25	r 2,0 pos.	r 18,4 pos.	r 22,5 pos.	r 2,0 pos.	r >25 pos.	r >25 pos.	
36	28	r 2,4 pos.	r 16,1 pos.	r 22,0 pos.	r 2,0 pos.	r >25 pos.	r >25 pos.	
38	28	r 2,1 pos.	r 17,9 pos.	r >25 pos.	r 2,1 pos.	r >25 pos.	r >25 pos.	
40	26	r 2,9 pos.	r >25 pos.	r >25 pos.	r 2,5 pos.	r >25 pos.	r >25 pos.	

1) marg. = marginally positive

1101 87002

Tab. 10 C

Animal No. 147

Time (weeks)	ALT	Commercial test (HP1)	4083 2 µg/ml	4083 0.5 µg/ml	4060 4081 2 µg/ml each	4074 4081 2 µg/ml each
--------------	-----	-----------------------	--------------	----------------	------------------------	------------------------

0 = inoculation	30	0,60 E ⁴⁹²	0,068 E ⁴⁵⁰	0,042 E ⁴⁵⁰	0,008 E ⁴⁵⁰	0,027 E ⁴⁵⁰
2	25	0,50 E	0,043 E	0,029 E	0,012 E	0,032 E
4	30	0,45 E	0,066 E	0,033 E	0,015 E	0,023 E

Extinctions (E)
define the
limits:

0,70 E	0,10 E	0,10 E	0,10 E	0,10 E
--------	--------	--------	--------	--------

ratios (r):

16	52	r 0,5	r 0,2	r 0,2	r 0,1	r 0,3
18	75	r 0,5	r 1,1 pos.	r 0,3	r 0,1	r 0,2
20	110	r 0,8	r 6,5 pos.	r 1,6 pos.	r 0,2	r 0,3
22	40	r 0,6	r 11,8 pos.	r 5,9 pos.	r 0,2	r 0,5
24	75	r 0,6	r 7,6 pos.	r 3,7 pos.	r 0,1	r 1,0 marg. ¹⁾
26	70	r 0,4	r 12,5 pos.	r 6,0 pos.	r 0,1	r 2,2 pos.
28	55	r 0,5	r 10,9 pos.	r 4,9 pos.	r 0,2	r 2,1 pos.
30	52	r 0,6	r 8,9 pos.	r 6,2 pos.	r 0,4	r 3,5 pos.
32	42	r 0,7	r 11,3 pos.	r 6,9 pos.	r 0,9	r 3,4 pos.
34	48	r 0,8	r 13,3 pos.	r 14,1 pos.	r 1,6 pos.	r 7,6 pos.
36	48	r 0,8	r 19,9 pos.	r 19,7 pos.	r 2,8 pos.	r 9,5 pos.
38	35	r 1,3 pos.	r 20,8 pos.	r 24,7 pos.	r 2,5 pos.	r 11,0 pos.
40	45	r 1,3 pos.	r >25 pos.	r >25 pos.	r 3,4 pos.	r 15,0 pos.

1) marg. = marginally positive

The results presented in Tab. 10 A - C underline the advantages of the peptides according to the invention, especially on comparison with the corresponding results from the commercial test (HP1) and the ALT course.

5 Thus, for example, animal No. 123 is not found to be
antibody-positive at all by commercial test (HP1)
although NANBH was detected by electron microscopy of
liver biopsies. By contrast, a reliable HCV antibody
10 detection is possible with the ELISA based on 4083 almost
at the same time as the ALT increase.

15 In particular, the chronological congruence of the
peptide ELISA results with the increased ALT levels is
evident for animal No. 048 where with all the described
peptides or peptide mixtures there is a very reliable, in
the sense of a sharp positive/negative discrimination,
anti-HCV detection almost at the same time as the ALT
increase and on average 18 weeks before the commercial
test (HP1). The comparisons among the peptides make it
particularly obvious that the central amino-acid sequence
20 makes a considerable contribution to the recognition of
the early HCV antibodies by the complete peptide of the
formula I (4083), as is made clear by the comparison of
the mixture of 4060 and 4081 (amino- and carboxyl-ter-
minal sequences) and 4090 which comprises the central
25 structure.

Similar results of an early identification of HCV anti-
bodies, almost simultaneously with ALT, were produced by
the investigations on animal No. 147 in which a reliable
HCV antibody detection is possible approximately simul-
30 taneously with the ALT increase, again about 16 - 18
weeks earlier than with the commercial test (HP1).

Tab. 11 A and B

5 Anti-HCV determination in serum samples from 2 chimpan-
zees infected with HCV. The results with the commercial
test (HP1) are presented in comparison with a syntheti-
cally prepared core peptide according to the invention
(SP 10, 2 μ g/ml), the results being presented as ratios
of specific signal and limit.

Animal No. 048

Time (weeks)	ALT	Commercial HCV ELISA (HP1)	SP 10-ELISA 2 µg/ml
--------------	-----	-------------------------------	------------------------

0 = inoculation

20	0,5 E ₄₉₂	0,09 E ₄₅₀
25	0,5 E	0,21 E
23	0,5 E	0,13 E

Extinctions

(E) define the

limits:

0,70 E

0,10 E

8	<u>ratios</u> (r)	34	r 0,4	0,50
10		32	r 0,6	r >20,0 <u>pos.</u>
12		100	r 0,4	r >20,0 <u>pos.</u>
14		112	r 0,7	r >20,0 <u>pos.</u>
16		30	r 1,0	r >20,0 <u>pos.</u>
18		25	r 0,7	r >20,0 <u>pos.</u>
20		30	r 0,8	r >20,0 <u>pos.</u>
22		25	r 0,8	r 19,0 <u>pos.</u>
24		23	r 0,8	r 20,0 <u>pos.</u>
26		20	r 0,8	r 18,5 <u>pos.</u>
28		20	r 0,8	r 10,4 <u>pos.</u>
30		20	r 1,0 <u>marg.¹⁾</u>	r 14,2 <u>pos.</u>
32		25	r 1,3 <u>pos.</u>	r 17,6 <u>pos.</u>
34		25	r 2,0 <u>pos.</u>	r 15,4 <u>pos.</u>
36		28	r 2,4 <u>pos.</u>	r >20,0 <u>pos.</u>
38		28	r 2,1 <u>pos.</u>	r >20,0 <u>pos.</u>
40		26	r 2,9 <u>pos.</u>	r >20,0 <u>pos.</u>

1) marg. = marginally positive

Animal No. 147

Time (weeks)	ALT	Commercial HCV ELISA (HP1)	SP 10-ELISA 2 µg/ml
--------------	-----	-------------------------------	------------------------

0 = inoculation

30	0,60 E ₄₉₂	0,011 E ₄₅₀
25	0,50 E	0,032
30	0,45 E	0,029

Extinctions

(E) define the

limits:

0,70 E

0,10 E

16	<u>ratios (r)</u>	52	r 0,5	r 0,2
18		75	r 0,5	r 0,1
20		110	r 0,8	r 0,2
22		40	r 0,6	r 0,4
24		75	r 0,6	r 0,3
26		70	r 0,4	r 0,2
28		55	r 0,5	r 0,3
30		52	r 0,6	r 0,2
32		42	r 0,7	r 0,4
34		48	r 0,8	r >20,0 <u>pos.</u>
36		48	r 0,8	not tested
38		35	r <u>1,3 pos.</u>	not tested
40		45	r <u>1,3 pos.</u>	r >20,0 <u>pos.</u>

1) marg. = marginally positive

The results presented in Tab. 11 A and B with a novel core peptide also underline the advantages of the peptides according to the invention, especially by comparison with the corresponding results of the commercial ELISA (HP1) and the ALT course.

In particular, the chronological congruence of the core peptide ELISA results with the increased ALT levels is clear for animal No. 048 where there is a very reliable, in the sense of a sharp positive/negative discrimination, anti-HCV detection almost at the same time as the ALT increase and on average 20 weeks before the commercial ELISA (HP1).

Similar results of an early identification, almost simultaneous with ALT, of HCV antibodies were produced by the investigations on animal No. 147 in which a reliable HCV antibody detection is in turn possible about 4 weeks earlier than with the commercial test (HP1).

Overall, the peptides according to the invention and the use thereof in immunochemical detection methods prove to be considerably more sensitive than all hitherto described methods based on proteins prepared by genetic engineering and other synthetic peptides of the state of the art. With improved sensitivity in later phases of infection, the novel peptides additionally provide considerable advantages in that reliable determination of early HCV antibodies is possible and thus the currently existing diagnostic gap is significantly reduced. Furthermore, the peptides according to the invention are considerably less sensitive to non-specific bindings, which is expressed not least by a drastic reduction in the background compared with the commercial test (HP1) (max. 0.10 E_{450} compared with max. 0.4 E_{492} with the commercial test (HP1)), both for samples of animal and of human origin so that a considerably sharper negative/positive discrimination is possible. Finally, advantageous aspects to be mentioned are the shorter overall

duration and the greater precision of determination owing to the sample volume of 50 μ l which can be pipetted more accurately.

Example 7:

- 5 Preparation of peptide solutions for producing a mixture of NSP 4 peptide 4083 and core peptide SP 10, and coating of microtiter plates with this peptide mixture

10 Serial 2-fold dilutions in 0.10 M sodium bicarbonate pH 9.6 were made up from stock solutions of the peptides SP 10 and 4083 in 50% acetic acid in distilled water each containing 6 mg of peptide/ml, that is to say a series with the concentrations 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, 0.1, 0.05 and 0.01 μ g of peptide/ml was obtained. The procedure was analogous for mixtures of
15 single peptides, the stock solutions additionally being mixed in various ratios, for example 10 : 1 or 1 : 4, in order to obtain by dilution in 0.1 M sodium bicarbonate the overall final concentrations which are indicated above but which in the case of mixtures of several peptides contain the latter in equal concentrations (in the case of 1 : 1 mixture) or in various ratios to one another.

20
25 In each case, 100 μ l of each dilution were placed in 16 wells of microtiter plates, type B supplied by Nunc, Roskilde, Denmark. The test plates charged with the dilutions were left at 20°C for 18 hours and then the solutions in the wells were removed by aspiration and the wells were washed 3 - 4 times with 300 μ l of a solution of 10 g/l bovine serum albumin in phosphate-buffered physiological saline (PBS, pH 7.4) by filling and removal
30 by aspiration, and the test plates were subsequently dried over silica gel at 20°C.

A concentration of 1 μ g of 4083/ml and 1 μ g of SP 10/ml has proven suitable for coating the peptide mixture, this

concentration representing the basis for the results which are summarized in Tab. 12 to 14 and were obtained as described in Examples 4 and 5. In contrast to previous comparisons with the state of the art, all subsequent comparisons will relate to a commercial anti-HCV ELISA of the 2nd generation (HP 2) as described in Example 3.

Tab. 12 and 13:

The data on the ELISA according to the invention and commercial HP 2 represent so-called end point titers with which the highest preliminary dilution, which was found to be reproducibly positive in a given test, of a serum in anti-HCV-negative serum is defined.

Tab. 12: BBI Low-titer anti-HCV PANEL PHV 101

MEMBER I.D. NUMBER	ELISA according to the invention SP 4083/SP 10 1 µg/ml each	Commercial anti- HCV test (HP2)
PHV101-01	1 : 16	1 : 2
PHV101-02	negative	negative
PHV101-03	1 : 4	1 : 1
PHV101-04	1 : 512	1 : 64
PHV101-05	1 : 2	1 : 1
PHV101-06	1 : 128	1 : 32
PHV101-07	1 : 128	1 : 128
PHV101-08	1 : 64	1 : 16
PHV101-09	1 : 64	1 : 16
PHV101-10	1 : 256	1 : 128
PHV101-11	1 : 32	1 : 16
PHV101-12	1 : 512	1 : 128
PHV101-13	1 : 16	1 : 64
PHV101-14	1 : 64	1 : 32
PHV101-15	1 : 128	1 : 64

Tab. 13: BBI Mixed-titer anti-HCV PANEL PHV 201

5

MEMBER I.D. NUMBER	ELISA according to the invention SP 4083/SP 10 1 µg/ml each	Commercial anti- HCV test (HP2)
PHV201-01	1 : 1000	1 : 128
PHV201-02	1 : 1000	1 : 500
PHV201-03	1 : 128	1 : 32
PHV201-04	negative	negative
PHV201-05	1 : 64	1 : 64
PHV201-06	1 : 64	1 : 256
PHV201-07	negative	negative
PHV201-08	1 : 4	1 : 16
PHV201-09	1 : 1000	1 : 512
PHV201-10	1 : 4	1 : 16
PHV201-11	1 : 512	1 : 128
PHV201-12	1 : 32	1 : 16
PHV201-13	1 : 256	1 : 256
PHV201-14	1 : 64	1 : 64
PHV201-15	1 : 512	1 : 1000
PHV201-16	1 : 512	1 : 512
PHV201-17	1 : 128	1 : 256
PHV201-18	1 : 16	1 : 128
PHV201-19	negative	negative
PHV201-20	1 : 4	1 : 4
PHV201-21	1 : 500	1 : 256
PHV201-22	1 : 16	1 : 256
PHV201-23	1 : 256	1 : 512
PHV201-24	1 : 256	1 : 512
PHV201-25	1 : 1000	1 : 512

Tab. 14:

Results of the tests on 930 healthy blood donors from whom serum and plasma were obtained simultaneously.

	Specificity in % after initial tests	Specificity in % after repeats
n = 930 sera	99.5	99.7
n = 930 plasmas	99.6	99.7

Example 8

Preparation of peptide solutions for producing mixtures according to formula XIV with peptides of the formulae XVIII, XIX, XX and XXII, and coating of microtiter plates with these peptide mixtures

The polypeptides SPH 9 (formula XVIII), SPH 20 (formula XIX), 4083 (formula XX) and SP 10 (formula XXII) were dissolved at a concentration of 6 mg/ml in 50% (v/v) acetic acid.

These 4 stock solutions were mixed in various volume-based ratios as described in Example 4 and diluted in 0.10 M sodium bicarbonate (pH 9.6) so that the total concentration of the polypeptides was between 0.2 and 8 µg/ml.

In each case 100 µl of each of the diluted solutions were placed in 16 wells of microtiter plates, type B supplied by Nunc, Roskilde, Denmark. The filled test plates were incubated at 20°C for 18 hours. The solutions were then removed by aspiration and the wells were washed 3 - 4 times with 300 µl of a solution of 10 g/l bovine serum albumin in phosphate-buffered physiological saline (PBS, pH 7.4), and the test plates were then dried over silica gel at 20°C.

Example 9

Optimization of the concentration of HIV 1, HIV 2 and HVC peptides for the coating mixture, and optimization of the ELISA determination and evaluation criteria

- 5 Starting with a ratio of 1 : 1 : 1 : 1 (V/V) for the four stock solutions described in Example 8, all four modifiable contents were varied independently of one another but keeping the three others constant in each case in order to obtain the following final concentrations of the individual peptides of HIV 1, HIV 2 and HCV in the coating solution (in µg/ml):
- 10

Peptide XVIII	Peptide XIX	Peptide XX	Peptide XXII
---------------	-------------	------------	--------------

2	2	2	2
1	2	2	2
0,5	2	2	2
0,2	2	2	2
0,1	2	2	2
0,05	2	2	2

Peptide XVIII	Peptide XIX	Peptide XX	Peptide XXII
---------------	-------------	------------	--------------

2	1	2	2
2	0,5	2	2
2	0,2	2	2
2	0,1	2	2
2	0,05	2	2
2	2	1	2

...

etc. to

0,05	0,05	0,05	0,05
------	------	------	------

5 These mixtures were immobilized as described in Example 8 on microtiter plates and evaluated in the ELISA as described in Example 4 and 5, with the concentration of the peroxidase-labeled antibody likewise being optimized against human immunoglobulin G.

10 The samples used to evaluate the optimization were composed of low-titer anti-HIV 1, anti-HIV 2 and anti-HCV samples which were prepared by serial dilutions of corresponding positive human sera in negative human serum. In addition, several anti-HIV and anti-HCV-negative samples were likewise tested in order to be able to define the background reaction (i.e. the non-specific binding on a given coated microtiter plate).

15 Set up as selection criterion was a signal of maximum specificity, i.e. high limiting sensitivity in the determination of the titration series of human anti-HIV- and anti-HCV-positive samples with, at the same time, a low background in the tests on anti-HIV- and anti-HCV-negative samples.

20 Coating mixtures which were usually favorable were found on the basis of these criteria, from which the following mixture was selected:

XXI SPH 9 0.500 μ g/ml
SPH 20 0.250 μ g/ml
25 SP 4083 0.500 μ g/ml
SP 10 0.125 μ g/ml

30 A conjugate concentration of Example 2 of 1 : 3000 with a uniform additional final dilution of 1 : 26 was found to be favorable, the test being carried out as in Example 4. In contrast to the previous establishment of limits, under these conditions samples having an extinction at 450 nm which is greater than the mean of the negative controls plus an addition of 0.250 O.D. were classified as anti-HIV- and/or anti-HCV-positive (new

establishment of limits).

These establishments were applied uniformly and unchanged to following Examples 10 to 15.

Example 10

- 5 Determination of human antibodies of the immunoglobulin G class against HIV 1, HIV 2 and HCV in the ELISA

The samples shown in Tab. 15 - 17 were tested as described in Example 4 under optimized conditions of Example 9 with the peptide mixture of the formula XIV.

- 10 With regard to anti-HIV 1 or 2, the reactivities of the method according to the invention were compared with an anti-HIV 1/2 combination test (Enzygnost^R anti-HIV 1/2, supplied by Behringwerke AG, HP 3). Commercially available Western blots (anti-HIV 1 and anti-HIV 2) supplied
15 by DuPont were used for control. HCV was detected with the aid of two different ELISA methods (HP 1 and HP 2).

As is made clear by the data in Tab. 15 to 17, anti-HIV 1 and anti-HIV 2, as well as anti-HCV, are detected safely and reliably in samples of human origin with the method according to the invention.

20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

Tab. 15

Determination of anti-HIV 1 with the method according to the invention compared with an anti-HIV 1/2 combination test (Enzygnost^R anti HIV 1/2, HP 3)

5 Strongly reactive means extinctions > 2.5 in the photometric evaluation; all 76 anti-HIV 1-positive samples are HCV-negative

10	Number of samples	Origin of samples	Method according to the invention	Commercial anti-HIV 1/2 combination test
15	n = 12 (anti-HIV 1 positive)	Europe	n = 12 strongly reactive	n = 12 strongly reactive
20	n = 57 (anti-HIV 1 pos.)	West Africa	n = 57 strongly reactive	n = 57 strongly reactive
25	n = 7 (HIV 1/ HIV 2 coinfections)	West Africa	n = 7 strongly reactive	n = 7 strongly reactive
30	n = 76		n = 76 positive	n = 76 positive

Tab. 16

Determination of anti-HIV 2 with the method according to the invention compared with an anti-HIV 1/2 combination test (Enzygnost^R anti HIV 1/2, HP 3)

5 Strongly reactive means extinctions > 2.5 in the photometric evaluation; all 28 anti-HIV 2-positive samples are HCV negative

10	Number of samples	Origin of samples	Method according to the invention	Commercial anti-HIV 1/2 combination test
15	n = 21 (anti-HIV 2 positive)	West Africa	n = 21 strongly reactive	n = 21 strongly reactive
20	n = 7 (HIV 1/ HIV 2 coinfections)	West Africa	n = 7 strongly reactive	n = 7 strongly reactive
25	n = 28		n = 28 positive	n = 28 positive

Tab. 17

Detection of anti-HCV with the ELISA method according to the invention

5 Samples were termed strongly reactive when they achieved
extinctions > 2.500. So-called ratios are stated for the
moderately strongly reactive samples, which means the
quotient of extinction of the sample to cut off of a
given ELISA. Values below 1.0 are, by agreement, termed
negative. Values above 1.0 as positive, the reactivity
10 being stronger as the resulting ratio increases; all
anti-HCV-positive samples are HIV-negative

Number of samples	Origin of the samples	Method according to the invention	Commercial anti- HCV ELISAs	
			HP 1	HP 2
15 n = 61	Europe	n = 57	n = 57	n = 57
Anti HCV positive		strongly reactive	strongly reactive	strongly reactive
	Europe	n = 4		
HC 90-346		> <u>9.0</u> ++	<u>2.7</u> (+)	> <u>5.0</u> ++
-351		<u>6.4</u> +	negative	<u>3.9</u> +
-516		<u>6.8</u> +	negative	<u>5.0</u> +
-570		<u>6.9</u> +	<u>1.9</u> (+)	> <u>5.0</u> +
n = 53	USA	n = 51	n = 51	n = 51
Anti HCV positive		strongly reactive	strongly reactive	strongly reactive
	USA	n = 2		
HC 90-511		<u>4.2</u> +	<u>1.2</u> (+)	<u>3.99</u> +
-566		<u>4.6</u> +	negative	> <u>5.00</u> +
n = 114		n = 114	n = 111	n = 114
		positive	positive	positive

Example 11:

Determination of the analytical sensitivity of the ELISA according to the invention for anti-HIV and anti-HCV compared with the state of the art

- 5 As a model for the assessment of the analytical sensitivity of the ELISA according to the invention, titrations of positive samples were prepared and tested in the ELISA described in Example 9. In total, dilutions of three each of anti-HIV 1- and anti-HIV 2-positive human sera in
10 anti-HIV- and anti-HCV-negative serum were prepared and the limiting sensitivity defined as the last preliminary dilution still reactive in the particular test system using the new limit (mean of the negative controls plus 0.250 threshold).
- 15 The results of these limiting sensitivity tests are compiled in Tab. 18 (anti-HIV 1), Tab. 19 (anti-HIV 2) and and Tab. 20 (anti-HCV) as extinctions.

Tab. 18

Determination of the limiting sensitivity of the ELISA according to the invention for anti-HIV 1 compared with the anti-HIV 1/2 combination test (Enzygnost[®] Anti-HIV 1/2).

Three confirmed anti-HIV 1-positive samples underwent preliminary serial two-fold dilution with human anti-HIV 1- and anti-HCV-negative sera, and these dilutions were tested as in Example 3 or in accordance with the pack insert of the manufacturer of the comparative test. All the data are extinctions (E_{450}). All anti-HIV-positive samples had a negative reaction in HP 2.

Sample	Preliminary dilution	Extinctions in the ELISA according to the invention	Extinctions in the anti-HIV 1/2 combination test (HP 3)
		cut off=0.282 O.D.	cut off=0.268 O.D.

8803/26	undiluted	> 2,500 +	> 2,500 +
	1 : 128	> 2,500 +	> 2,500 +
Anti	256	2,334 +	2,185 +
HIV 1-	512	1,408 +	1,412 +
positive	1024	0,825 +	0,837 +
	2048	<u>0,467</u> +	<u>0,457</u> +
	4096	0,240 -	0,216 -
	8000	0,137 -	0,135 -

8611/144	undiluted	> 2,500 +	> 2,500 +
	1 : 128	2,162 +	2,014 +
Anti	256	1,366 +	1,028 +
HIV 1-	512	0,806 +	0,648 +
positive	1024	<u>0,496</u> +	<u>0,454</u> +
	2048	0,280 -	0,258 -
	4096	0,153 -	0,136 -
	8000	0,088 -	0,076 -

8803/24			
Anti	1 : 400	<u>1,632</u> +	<u>1,585</u> +
HIV 1-			
positive			

22281			
Anti HCV-	1 : 256	<u>2,219</u> +	0,014
positive			(negative)

Tab. 19

Determination of the limiting sensitivity of the ELISA according to the invention for anti-HIV 2 compared with the anti-HIV 1/2 combination test (Enzygnost^R Anti-HIV 1/2).

Three confirmed anti-HIV 2-positive samples underwent preliminary serial two-fold dilution with human anti-HIV 1- and anti-HCV-negative sera, and these dilutions were tested as in Example 3 or in accordance with the pack insert of the manufacturer of the comparative test. All the data are extinctions (E_{450}). All anti-HIV-positive samples had a negative reaction in HP 2.

Sample	Preliminary dilution	Extinctions in the ELISA according to the invention	Extinctions in the anti-HIV 1/2 combination test (HP 3)
		cut off=0.282 O.D.	cut off=0.268 O.D.

8804/103	undiluted	> 2,500 +	> 2,500 +
	1 : 128	> 2,500 +	2,500 +
Anti	256	2,420 +	2,045 +
HIV 2-	512	1,488 +	1,339 +
positive	1024	0,737 +	0,705 +
	2048	<u>0,365</u> +	<u>0,385</u> +
	4000	0,196 -	0,190 -

8804/117	undiluted	> 2,500 +	> 2,500 +
	1 : 128	> 2,500 +	> 2,500 +
Anti	256	1,860 +	1,902 +
HIV 2-	512	1,233 +	1,100 +
positive	1024	0,656 +	0,667 +
	2048	<u>0,320</u> +	<u>0,302</u> +
	4000	0,166 -	0,172 -

8804/116			
Anti	1 : 400	<u>1,045</u> +	<u>1,020</u> +
HIV 2-			
positive			

22281			
Anti HCV-	1 : 256	<u>2,219</u> +	0,014
positive			(negative)

Tab. 20

Determination of the limiting sensitivity of the ELISA according to the invention for anti-HCV.

5 Four confirmed anti-HCV-positive samples underwent preliminary serial two-fold dilution with human anti-HIV- and anti-HCV-negative sera, and these dilutions were tested as in Example 3. All the data are extinctions ($E_{450 \text{ nm}}$).

10 Sample	Preliminary dilution	Extinctions in the ELISA according to the invention cut off=0.270	Commercial anti-HCV ELISA (HP 2) cut off=0.481
HC 90-85	undiluted	> 2,500 +	> 2,500 +
	1 : 16	> 2,500 +	> 2,500 +
	1 : 32	2,299 +	> 2,500 +
	1 : 64	1,858 +	> 2,500 +
	1 : 128	1,515 +	> 2,500 +
	1 : 256	1,045 +	1,707 +
	1 : 512	0,648 +	0,871 +
	1 : 1024	<u>0,322</u> +	<u>0,492</u> +
	1 : 2048	0,207 -	0,255 -
Anti HCV-positive	undiluted	> 2,500 +	> 2,500 +
	1 : 16	> 2,500 +	> 2,500 +
	1 : 32	2,214 +	> 2,500 +
	1 : 64	1,816 +	> 2,500 +
	1 : 128	1,488 +	2,008 +
	1 : 256	0,689 +	1,120 +
	1 : 512	0,580 +	<u>0,501</u> +
	1 : 1024	<u>0,286</u> +	0,375 -
	1 : 2048	0,148 -	0,111 -
Anti. HCV-positive			
HC 90-270	1 : 256	<u>0,999</u> +	<u>1,544</u> +
HC 90-354	1 : 128	<u>0,971</u> +	<u>0,761</u> +
Anti HIV 1-positive			
8803/26	undiluted	> <u>2,500</u> +	0,110 -
8811/144	undiluted	> <u>2,500</u> +	0,098 -
8803/24	undiluted	> <u>2,500</u> +	0,068 -
Anti HIV 2-positive			
8804/103	undiluted	> <u>2,500</u> +	0,075 -
8804/117	undiluted	> <u>2,500</u> +	0,081 -
8804/116	undiluted	> <u>2,500</u> +	0,099 -
(negative)			
(negative)			

The procedure was analogous for anti-HCV in that serial dilutions of four anti-HCV-positive samples were prepared as described in Tab. 20, tested and evaluated in analogy to anti-HIV and compared with the results with the commercial anti-HCV ELISA of the 2nd generation (HP 2).

In addition, the specificity of the reactivities of individual donations were also tested in the individual tests by also investigating the anti-HIV 1- and anti-HIV 2-positive samples undiluted in the anti-HCV test and, conversely, the anti-HCV-positive samples in anti-HIV 1/2 combination tests (Enzygnost^R anti-HIV 1/2).

The results compiled in Tab. 18 to 20 make it clear that the limiting sensitivity of the ELISA according to the invention for both anti-HIV 1 and anti-HIV 2 corresponds to the limiting sensitivity of the anti-HIV 1/2 combination test. The HCV limiting sensitivity of the ELISA according to the invention also corresponded to the limiting sensitivity of a commercially available anti-HCV ELISA (HP 2). However, while a total of at least two tests (anti-HIV 1/2 combination test and at least one anti-HCV test) are necessary to detect these three antibody specificities according to the state of the art, this detection is possible with the method according to the invention just as reliably and with comparable sensitivity using only one test mixture.

It is furthermore evident from the data in Tab. 18 to 20 that the strong reactivity of the anti-HIV- and anti-HCV-positive samples in the novel ELISA is particularly advantageous because the anti-HIV samples reacted negatively in the specific anti-HCV test and the anti-HCV-positive samples reacted negatively in the specific anti-HIV test.

Example 12

Determination of anti-HIV 1-positive samples from early stages of infection (seroconversions) with the ELISA according to the invention.

5 Tests were carried out on a total of 3 patients from whom sequential blood samples were repeatedly obtained at defined times during very early phases of an HIV 1 infection. These serum panels are commercially available (Boston Biomedica Inc., USA). The results which were
10 obtained with the ELISA according to the invention compared with an anti-HIV 1/2 combination test have been reproduced in Tab. 21.

Tab. 21:

15 All data are extinctions ($E_{450 \text{ nm}}$), and values above the particular limit are to be regarded as positive.

Tab. 21

Sample code	ELISA according to the invention	Commercial anti-HIV 1/2 ELISA	Commercial anti-HCV ELISA (HP2)
1 A	0,023	0,066	negative
2 A	0,020	0,055	"
3 A	0,055	0,193	"
4 A	1,163 +	> 2,500 +	"
5 A	> 2,500 +	> 2,500 +	"
6 A	> 2,500 +	> 2,500 +	"
7 A	> 2,500 +	> 2,500 +	"
8 A	> 2,500 +	> 2,500 +	"
9 A	> 2,500 +	> 2,500 +	"
1 C	0,014	0,022	negative
2 C	0,015	0,054	"
3 C	0,054	0,145	"
4 C	0,500 +	1,138 +	"
5 C	0,630 +	1,707 +	"
6 C	0,889 +	> 2,500 +	"
7 C	0,942 +	"	"
8 C	1,010 +	"	"
9 C	1,081 +	"	"
10 C	1,564 +	"	"
11 C	> 2,500 +	"	"
12 C	"	"	"
13 C	"	"	"
14 C	"	"	"
15 C	"	"	"
16 C	"	"	"
17 C	"	"	"
18 C	"	"	"
G BBI			
80	0,012	0,056	negative
81	0,013	0,053	"
82	0,018	0,059	"
83	0,478 +	1,112 +	"
84	0,733 +	> 2,500 +	"
85	0,612 +	> 2,500 +	"
86	0,763 +	> 2,500 +	"
87	1,296 +	> 2,500 +	"
88	1,309 +	> 2,500 +	"
89	1,544 +	> 2,500 +	"
cut off	0,281	0,285	0,481

The results in Tab. 21 make it clear that the ELISA according to the invention detects low-titer anti-HIV 1-positive samples just as reliably as the anti-HIV 1/2 combination test (Enzygnost^R anti-HIV 1/2). It is likewise evident from comparison with the data in Tab. 21 that the earliest time at which first detection of HIV 1-specific antibodies is possible with the novel peptide ELISA is also possible at least as early as with specific single anti-HIV 1 tests as state of the art.

10 Example 13

Determination of low-titer anti-HIV 1-positive samples with the ELISA according to the invention

15 Tab. 22 summarizes the results which were obtained in the tests on a commercially available anti-HIV low-titer panel with the novel HIV/HCV peptide ELISA compared with an anti-HIV 1/2 combination test (Enzygnost^R anti-HIV 1/2).

20 The sample panel supplied by Boston Biomedica; Inc. , USA, comprises a total of 15 samples which were classified by means of anti-HIV 1 determination methods as low-titer anti-HIV 1-positive.

Tab. 22

Results of the tests on the low-titer anti-HIV 1 panel supplied by Boston Biomedica Inc., USA

25 All the data are extinctions at a wavelength of 450 nm. Values above the stated limits are positive.

Tab. 22

ID No.	ELISA according to the invention		Commercial anti-HIV 1/2	Commercial anti-HCV
	ELISA	HCV	ELISA	
BO 1-01	> 2,500	1,872	> 2,500 +	
02	1,979	1,575	2,023 +	
03	2,056	2,280	negative	
04	1,751	1,960	"	
05	1,781	2,044	"	
06	1,428	1,728	"	
07	1,183	1,564	"	
08	0,641	1,152	"	
09	0,984	1,499	"	
10	0,682	0,940	"	
11	1,302	1,650	"	
12	2,032	0,540	1,833 +	
13	1,017	1,854	negative	
14	1,079	1,484	"	
15	> 2,500	2,090	> 2,500 +	
cut off	0,279	0,265	0,479	

The results in Tab. 22 reveal that, compared with the anti-HIV 1/2 combination test (Enzygnost^R anti-HIV 1/2), all the samples are found to be comparably strongly reactive with the HIV/HCV peptide ELISA according to the invention.

It is interesting that four samples in the panel are in fact found to be more strongly reactive with the novel peptide ELISA than with the anti-HIV 1/2 combination test (Enzygnost^R anti-HIV 1/2) (No. 1, 2, 12 and 15). Additional tests on these samples revealed the simultaneous presence of HCV antibodies, which again confirms the reliability of a non-differentiating detection of anti-HIV 1/-HIV 2 and -HCV by the method according to the invention.

Example 14

Determination of low-titer anti-HCV-positive samples with the ELISA according to the invention

Tab. 23 contains the results which were obtained with the novel HIV 1/HIV 2 and HCV peptide ELISA and the low-titer anti-HCV panel supplied by Boston Biomedica Inc., USA. This Tab. 23 also contains data which were obtained on the same panel with a modern anti-HCV ELISA (HP 2).

The data on the ELISA according to the invention and commercial represent so-called end point titers with which the highest preliminary dilution, which was found to be reproducibly positive in a given test, of a serum in anti-HCV-negative serum is defined.

Tab. 23: BBI low-titer anti-HCV PANEL PHV 101

MEMBER I.D. NUMBER	ELISA according to the invention	Commercial anti- HCV test (HP 2)
PHV101-01	1 : 16	1 : 2
PHV101-02	negative	negative
PHV101-03	1 : 4	1 : 1
PHV101-04	1 : 512	1 : 64
PHV101-05	1 : 2	1 : 1
PHV101-06	1 : 128	1 : 32
PHV101-07	1 : 128	1 : 128
PHV101-08	1 : 64	1 : 16
PHV101-09	1 : 64	1 : 16
PHV101-10	1 : 256	1 : 128
PHV101-11	1 : 32	1 : 16
PHV101-12	1 : 512	1 : 128
PHV101-13	1 : 16	1 : 64
PHV101-14	1 : 64	1 : 32
PHV101-15	1 : 128	1 : 64

The results in Tab. 23 make it clear that all 14 positive samples were found reliably and unambiguously to be positive with the ELISA according to the invention. It is noteworthy in this connection that the signal strength is very high, which is attributable to high reactivity.

This high reactivity is also reflected in the limiting sensitivities found in Tab. 23. These detection limits were defined by subjecting the native human samples to preliminary serial two-fold dilutions in anti-HCV-negative serum and then to testing as in Example 4, where the last preliminary dilution stage which is still found to be reactive represents the so-called final titer.

On this basis, the data in Tab. 23 reveal that the novel HIV 1/HIV 2/HCV peptide ELISA has in fact better detection limits for anti-HCV in the PHV 101 panel than the state of the art.

Example 15

Determination of the rate of non-specific reactions of the novel peptide ELISA in a group of healthy blood donors

To determine the frequency of non-specific reactions, which lead to false positive results in the ELISA, a total of $n = 512$ healthy blood donors was used. Serum and plasma were obtained simultaneously from these donors in order to be able also to investigate a possible interfering effect of the coagulation system on the method.

Samples which were reactive in one of these three tests (ELISA according to the invention, anti-HCV ELISA HP 2 and anti-HIV 1/2 HP 3) (so-called initial tests) were repeated in the same test (so-called retesting) and, where the reactivity was reproducible, investigated in the anti-HIV 1 Western blot and anti-HCV ELISA (HP 1) as comparative methods.

It was not possible to confirm as anti-HIV 1- or as anti-HCV-positive by means of this confirmatory method any of the samples which emerged in this screening as reactive in one of the three ELISAs. Thus, all the samples which were rejected in the screening were consistently classified as false-positive in the particular methods.

Tab. 24:

Screening of n = 512 healthy blood donors from whom 512 sera and 512 so-called paired plasma were obtained simultaneously. The findings after the initial tests are indicated; see Tab. 25 for the retest results.

111 91

87002

Tab. 24

Sample Code	Method according to the invention		Anti-HIV 1/2		Anti-HCV (HP 2)	
	<u>Number</u>	<u>ratios</u>	<u>Number</u>	<u>ratios</u>	<u>Number</u>	<u>ratios</u>
	reactive sera/ plasmas	sera/ plasmas	reactive sera/ plasmas	sera/ plasmas	reactive sera/ plasmas	sera/ plasmas
	3 / 2		0 / 0		0 / 0	
9104-PE-1		2,1 / 1,3		neg.		neg.
2		1,0 / 1,2		neg.		neg.
3		1,1 / 0,9 -		neg.		neg.
	0 / 0		1 / 1			
9104-HIV-1		neg.		1,8 / 1,6		neg.
	0 / 0		0 / 0		5 / 4	
9104-HCV-1		neg.		neg.		>4,0 / 3,5
2		neg.		neg.		2,5 / 3,0
3		neg.		neg.		1,4 / 1,2
4		neg.		neg.		1,3 / 1,5
5		neg.		neg.		0,8 - / 1,1

11191 87002

Tab. 25 Results of the specificity after repeat tests on the 9 reactive sera from Tab. 24

n = 512 sera and n = 512 paired plasmas from a total of 512 healthy blood donors	novel peptide ELISA	anti-HIV 1/2 (HP 3)	anti-HCV (HP 2)
	serum / plasma	serum / plasma	serum / plasma
Number of false- positive results in initial tests	3 / 2	1 / 1	5 / 4
initial specificity (%)	99.41 / 99.60	99.80 / 99.80	99.02 / 99.22
Number of false- positive results after repeat	2 / 2	1 / 1	4 / 4
retest specificity (%)	99.60 / 99.60	99.80 / 99.80	99.22 / 99.22

The results in the first series of tests (initial results) are compiled in Tab. 24. Whereas three sera (and two corresponding plasmas) were reactive in the method according to the invention, 1 serum (and one corresponding plasma) was found to be reactive with an anti-HIV 1/2 ELISA and five sera (and four corresponding plasmas) were found to be reactive with the anti-HCV ELISA. The donors reactive in the particular ELISA were not identical.

After repeat tests had been carried out on these nine reactive sera, the picture which emerged was that depicted in Tab. 25 that two sera (two paired plasmas) were retest reactive in the peptide ELISA according to the invention, one serum (one paired plasma) was reactive in the anti-HIV 1/2 ELISA, and four sera (four paired plasmas) were reactive in the anti-HCV ELISA.

On the basis that it was not possible to confirm any of these samples as anti-HIV- and/or anti-HCV-positive, the specificity data presented in Tab. 25 were determined for each of the ELISAs investigated and for sera and plasmas by calculating the percentage of samples found correctly to be negative (in total 512 sera and 512 plasmas).

Based on the data in Tab. 25, according to the state of the art - obligatory tests for anti-HIV and anti-HCV on each donation, a total of five donors would have had to be excluded:

One donor repeatably false-positive in the anti-HIV 1/2 test and four donors repeatably false-positive in the anti-HCV test. By contrast, the number of donors possibly found with a false reaction is only two in the ELISA according to the invention.

In summary, it can be said that the novel peptide ELISA for the simultaneous non-differentiating detection of anti-HIV 1, anti-HIV 2 and anti-HCV at least corresponds in terms of the currently valid criteria of sensitivity to the in each case optimal performance features of the

two single tests anti-HIV 1/2 on the one hand and anti-HCV on the other hand: the data from the tests on panels, i.e. native samples containing anti-HIV 1 and/or anti-HIV 2 and/or anti-HCV indicate comparable efficiency just like the tests on the limiting sensitivities and time of earliest recognition of low-titer anti-HIV 1 or anti-HIV 2 or anti-HCV samples. Whereas detection of these three antibody specificities is possible according to the state of the art in this manner only with three different or, in view of the anti-HIV 1/2 combination tests, with two different tests, the ELISA according to the invention has the advantage that, while the efficiency is the same, only one test need be carried out. Particularly for blood banks which are obliged to carry out tests for anti-HIV 1, anti-HIV 2 and anti-HCV, the novel peptide ELISA means a considerable reduction in effort and costs with at least the same reliability and safety in the determination of anti-HIV 1, anti-HIV 2 and anti-HCV.

It was additionally found, completely surprisingly, when determining the susceptibility of this novel test to interference that, by reason of the very good specificity, i.e. only low number of false-positive findings, fewer retests and fewer confirmatory tests are necessary and, moreover, fewer donations have to be rejected than with the conventional screening procedure using an anti-HIV 1/2 test and, separate therefrom, an anti-HCV test. This means that the method according to the invention has economic and chemical advantages which are also offered by other constellations of HIV and HCV peptides of the formulae IV to XII and XIII to XVII.

The method according to the invention is also extremely suitable for other problems in which maximal optimization of limiting sensitivity is of most importance. Thus, it is possible to show under other test conditions or, for example, by calculation in Examples 10 to 14 that it is perfectly possible to produce sensitivity features of the method according to the invention which are significantly

better than those of the state of the art if somewhat less favorable specificity data are accepted, although these still correspond to the state of the art.

5 If, for example, the limit (Example 9) were to be reduced to 0.1 extinction, all the limiting sensitivities for anti-HIV 1, anti-HIV 2 and anti-HCV would be considerably improved by a factor of at least 2. A total of five donors would result as the rate of samples with a non-specific, i.e. false-positive, reaction in the screening
10 of the healthy blood donors (Example 15), the donors having been found to be repeatedly reactive, which corresponds absolutely to the result of the two single tests, although the sensitivity of the antibody detection was considerably improved.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A mixture of peptides composed of at least one of the peptides of group A which group consists of the peptides:

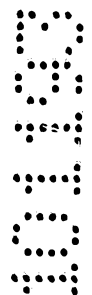
1	30	
MSTNPKPQRKTKRNTNRRPQDVKFPGGGQI		SP 10
8	26	
QRKTKRNTNRRPQDVKFPG		SP 23
10	26	
KTKRNTNRRPQDVKFPG		SP 30
12	26	
KRNTNRRPQDVKFPG		SP 30 D
14	26	
NTNRRPQDVKFPG		SP 30 C
16	26	
NRRPQDVKFPG		SP 30 B
18	26	
RPQDVKFPG		SP 30 A
8	24	
QRKTKRNTNRRPQDVKF		SP 31
8	22	
QRKTKRNTNRRPQDV		SP 32

and at least one of the peptides of group B (non-structural protein of HCV), which group consists of the peptides:

4083:

121

SGKPAIIPDREVLVREFDEMEECSQHLPYIEQGMMLAEQFKQKALGLLQTASRQA



91

4074:

121 160
SGKPAIIPDREVLRYREFDEMEECSQHLPYIEQGMLAEQF

4073:

128 160
PDREVLRYREFDEMEECSQHLPYIEQGMLAEQF

4072:

136 160
EFDEMEECSQHLPYIEQGMLAEQF

4071:

144 160
SQHLPYIEQGMLAEQF

4081:

161 175
KQKALGLLQTASRQA

4082:

144 175
SQHLPYIEQGMLAEQFKQKALGLLQTASRQA

4090:

135 164
REFDEMEECSQHLPYIEQGMLAEQFKQKA

4056:

121 135
SGKPAIIPDREVLRY

4055:

129 143
DREVLRYREFDEMEEC



92

4054:

137

151

FDEMEECSQHLPYIE

4053:

145

159

QHLPYIEQGMMLAEQ

4052:

153

167

GMMLAEQFKQKALGL

4060:

121

139

SGKPAIIPDREVLYREFDE

2. The mixture of claim 1 consisting of

SP 10

MSTNPKPQRKTKRNTNRRPQDVKFPGGGQI

and

4083

121

175

SGKPAIIPDREVLYREFDEMEECSQHLPYIEQGMMLAEQFKQKALGLLQT

ASRQA.

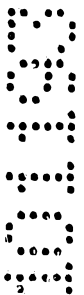
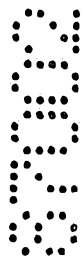
3. Mixture of claims 1 and 2 characterized in that the amino acid sequence of the peptides has been modified in comparison to the natural sequences by replacement or deletion of one or more amino-acids in such a way that the immunomedical reactivity is maintained or improved.

4. The mixture as claimed in at least one of claims 1 to 3, wherein the peptides are linked to one another directly or via carrier.

5. The mixture of at least one of claims 1 to 4, wherein the peptides are immobilized to a solid support.



6. The mixture of claim 1, wherein the peptides have been synthesized by peptide chemistry.
7. The mixture of at least claims 1 to 6 characterized in that it contains additional HIV peptides, which may be recombinant or synthetic and may be combined with ~~recombinant and synthetic~~ peptides according to claim 6.
8. ^{DNA}
A _^sequence, which codes for the peptides of a mixture according to at least one of the claims 1 to 5.
9. Antibody having a specific affinity for a mixture of peptides according to a least one of the claims 1 to 6.
10. Immunochemical method for the detection and/or determination of antibodies against HCV, wherein a mixture of peptides according to at least one of claims 1 to 7 is used.
11. Analytical method for the detection and determination of HCV ^{comprising} ~~characterized in that the specific step is a hybridization reaction wherein a probe is used which in its specific part~~ is complementary to a DNA sequence according to claim 8.
12. The method of claim 10, wherein the mixture of peptides is immobilized on a carrier.
13. The method of claim 12, wherein the carrier is selected from the group comprising polystyrene, polyvinyl chloride, polyamide and other synthetic polymers, natural polymers such as cellulose and derivatized natural polymers such as cellulose acetate and nitrocellulose, as well as glass, especially as glass fibers.
14. The method of claim 13, wherein the carrier is polystyrene.



- for the detection and/or determination of HCV antibodies
15. The method ~~as claimed in claim 21~~, wherein the detection and/or the determination of an epitope-bound antibody is carried out with the aid of enzyme-labeled, fluorescence-labeled, chemiluminescence-labeled, biotin-labeled or radioactively labeled antibodies against the epitope-bound antibodies.
16. Method of claim 15, wherein the enzymes alkaline phosphatase and/or horseradish peroxidase are used as enzyme labels for the antibodies.
17. The method of claim 12, characterized in that the "Immuno-Dot" method is used.
18. The method of using mixtures of peptides as claimed in at least one of claims 1 to 7 for raising antibodies in mammals, especially in humans.
19. The use of the antibodies as claimed in claim 9 for diagnostic and therapeutic purposes.
20. The use of antibodies as claimed in claim 19, wherein the diagnostic method is a heterogeneous immunoassay.
21. ~~An immunochemical test kit for the detection and/or determination of HCV antibodies, where the test kit contains one or more carriers on which peptide mixtures as claimed in at least one of claims 1 to 7 are immobilized.~~

DATED this 9th day of November, 1993.

BEHRINGWERKE AKTIENGESellschaft

WATERMARK PATENT & TRADEMARK ATTORNEYS
THE ATRIUM
290 BURWOOD ROAD
HAWTHORN VICTORIA 3122
AUSTRALIA



Sequence listing:

HOE 90/B 032K

Sequence No. 1

HCV amino-acid sequence

121

175

SGKPAIIPDREVLRYREFDEMEECSQHLPLYIEQGMMMLAEQFKQKALGLLQTASRQA

Sequence No. 2

HCV amino-acid sequence

121

160

SGKPAIIPDREVLRYREFDEMEECSQHLPLYIEQGMMMLAEQF

Sequence No. 3

HCV amino-acid sequence

128

160

PDREVLRYREFDEMEECSQHLPLYIEQGMMMLAEQF

Sequence No. 4

HCV amino-acid sequence

136

160

EFDEMEECSQHLPLYIEQGMMMLAEQF

Sequence No. 5

HCV amino-acid sequence

144

160

SQHLPLYIEQGMMMLAEQF

Sequence No. 6

HCV amino-acid sequence

161

175

KQKALGLLQTASRQA

Sequence No. 7

HCV amino-acid sequence

144

175

SQHLPYIEQGMMMLAEQFKQKALGLLQTASRQA

Sequence No. 8

HCV amino-acid sequence

135

164

REFDEMEECSQHLPYIEQGMMMLAEQFKQKA

Sequence No. 9

HCV amino-acid sequence

121

135

SGKPAIIPDREVLJR

Sequence No. 10

HCV amino-acid sequence

129

143

DREVLJREFDEMEEC

Sequence No. 11

HCV amino-acid sequence

137

151

FDEMEECSQHLPYIE

Sequence No. 12

HCV amino-acid sequence

145

159

QHLPYIEQGMMMLAEQ

Sequence No. 13
HCV amino-acid sequence

153 167
GMMLAEQFKQKALGL

Sequence No. 14
HCV amino-acid sequence

345 359
VQWMNRLLIAFASRG

Sequence No. 15
HCV amino-acid sequence

121 139
SGKPAIIPDREVLVREFDE

Sequence No. 16
HCV amino-acid sequence.

HVGPGEAVQWMNRLLIAFASRGNHVSP

Sequence No. 17
HCV amino-acid sequence

MSTNPKPQRKTKRNTNRRPQDVKFPGGQIVGGVY I

Sequence No. 18
HCV amino-acid sequence

1 30
MSTNPKPQRKTKRNTNRRPQDVKFPGGQI SP 1.0

Sequence No. 19
HCV amino-acid sequence

8 26
QRKTKRNTNRRPQDVKFPG SP 2.3

Sequence No. 20
HCV amino-acid sequence

10 26
KTKRNTNRRPQDVKFPG

SP 30

Sequence No. 21
HCV amino-acid sequence

12 26
KRNTNRRPQDVKFPG

SP 30 D

Sequence No. 22
HCV amino-acid sequence

14 26
NTNRRPQDVKFPG

SP 30 C

Sequence No. 23
HCV amino-acid sequence

16 26
NRRPQDVKFPG

SP 30 B

Sequence No. 24
HCV amino-acid sequence

18 26
RPQDVKFPG

SP 30 A

Sequence No. 25
HCV amino-acid sequence

8 24
QRKTKRNTNRRPQDVKF

SP 31

Sequence No. 26
HCV amino-acid sequence

8 22
QRKTKRNTNRRPQDV

SP 32

Sequence No. 27
HCV amino-acid sequence

-DVEVLYR-

Sequence No. 28
HCV amino-acid sequence

-QHLPYIE-

Sequence No. 29
HCV amino-acid sequence

-KQKALGL-

Sequence No. 30
HCV amino-acid sequence

~QRKTKRNTNRRPQDVK-

Sequence No. 31
HIV amino-acid sequence

586 620
RILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS

Sequence No. 32
HIV amino-acid sequence

578 613
RVTAIEKYLQDQARLNSWGCAFRQVCHTTVPWVND

Fig. 1**Amino acid sequence of the ORF-region of C-100-3**aa 1

DAHFLSQTKQSGENLPYLV A	20
YQATVCA RAQA PPSWDQM W	40
KCLIRL KPTLHGPTPLLYRL	60
GAVQNEITLTHPVTKYIMTC	80
MSADLEVVTSTWVLVGGLA	100

ALAA YCLSTGCVVIVGRVVL	120
SGKPAIIPDREVL YREFDEM	140
EECSQHLPYIEQGMM LAEQF	160
KQKALGLLQTASRQA EVIAP	180
AVQTNWQKLETFWAKH MWNF	200

ISGIQYLAGLSTLPGNPAIA	220
SLMAFTA AVTSPLTTSQTLL	240
FNILGGWVAAQLAAPGAATA	260
FVGAGLAGAAIGSVGLGKVL	280
IDILAGYGAGVAGALVAFKI	300

MSG EVPSTEDLVNLLPAILS	320
PGALVVGVVCAAILRRHVGP	340
GEGAVQWMNRLIAFASRGNH	360
VSP	

aa 363

Fig. 2: Comparison of the amino acid sequences according to formula I and II of with peptides from the ORF-region of C-100-3

aa 1 [REDACTED] aa 363

298 362 sp 65

Sequences according to the invention

337 363

Formula I

Formula II

1 1

