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(54) Title: AN IMMUNOGENIC VACCINE AGAINST THE HCV AND/OR HBV

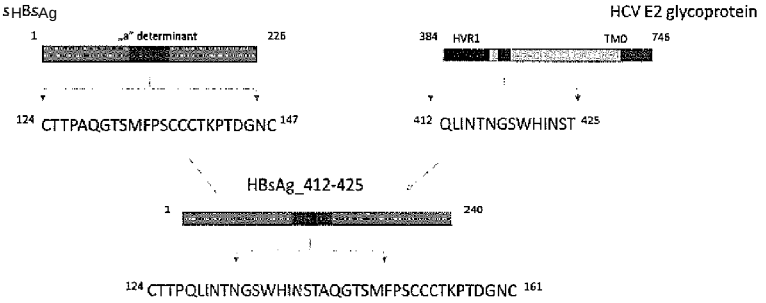

Fig. 1

(57) Abstract: The invention concerns a vaccine containing determinant 'a' of the HBsAg protein of the HBV virus, subtype adw2, and region 412-425 of E2 envelope glycoprotein of the HCV. The invention further concerns isolated chimeric protein in the form of vaccine, a combination containing chimeric protein, and a method of obtaining the vaccine. Furthermore, the invention concerns a composition containing chimeric protein and an expression vector.

An immunogenic vaccine against the HCV and/or HBV

The invention concerns an immunogenic vaccine designated for administration in prophylaxis of the HCV and/or HBV viral infections, and the method of obtaining it. The invention further concerns chimeric protein and a combination containing the vaccine. Moreover, the invention concerns a combination containing chimeric protein and an expression vector.

About 170 million individuals in the world are infected with the Hepatitis C Virus (HCV), which represents about 2-3% of the global population. The highest infection rate is found in Egypt where the HCV-infected account for as much as 20% of the population. The tests conducted all over Poland reveal that the sub-population of individuals infected with the Hepatitis C Virus ranges between 1.9 and 4%. However, because no attempt has been taken to hold tests which would embrace the entire population and gauge the actual scale of the problem, the number of the infected is only estimated at about 700 thousand. The modes of transmitting HCV infection are varied. As early as at the outset of HCV research and investigations a note was made of the observed relationship between the symptoms of the disease and the patient's prior contact with blood or blood derivatives. Today, thanks to the introduced hospital procedures developed countries rarely record HCV infections during medical procedures. In the developing countries, however, they continue to rank among the most frequent modes of transmission. As for the developed countries, the most common modes of infection include intravenous injection drug use (about 60% of new infections), mother-to-child virus transmission, and sexual contacts. Considering the astounding variety of human behaviours which carry the risk of coming in contact with blood or blood derivative products, one can list many more activities potentially carrying the risk of HCV infection. Let us but name e.g. needle-involving cosmetic procedures such as piercing, tattooing, or acupuncture. Most of such procedures are performed in regions where no routine screening tests for HCV infection are held; hence it is difficult to gauge their impact on the spreading of the epidemic. About 80% of the HCV infections become chronic, where no specific symptoms may occur for years, and for this reason HCV infection is frequently referred to as 'silent epidemic'. 20% of chronic infections lead to severe liver damage, hepatic cirrhosis, and consequently the development of the hepatocellular carcinoma. Frequently, the last resort option left for the patients is liver transplantation, though this, unfortunately, does not guarantee the

cure. The transplanted liver is immediately infected with the virus particles circulating in the blood or nested in cells other than those of the liver. Within the time ranging between several and several dozen days after the transplantation the patients are recorded to succumb to increased viremia. For years, the standard anti-viral treatment consisted in a combination therapy with Interferon and Ribavirin. Unfortunately, the therapy carried severe side effects and its effectiveness did not exceed 50%. Intense research effort of the recent years has broadened our knowledge of the biology of the HCV and in effect has led to the discovery of medicines acting directly on the virus particles (direct acting antivirals - DAAs). Available today, are drugs acting on both the viral proteases such as Telaprevir or Boceprevir, and on the RNA polymerase, e.g. Sofosbuvir; the effectiveness of the latter reaches as high as 90%. Unfortunately, the prices of the drugs, just as the cost of hospitalising the infected, are very high and impose a substantial burden on both the patients and the state. Considering all of the above aspects, development of an effective and commonly available vaccine which would protect against the infection is a major goal of the current HCV research.

HCV is a relatively small enveloped virus of the *Flaviviridae* family. Its genetic material is single-stranded RNA of positive polarity. The virus genome is associated with the multimeric protein core. The envelope forming the outer part of the virus is a lipid membrane with the protective E1 and E2 glycoproteins, forming the noncovalent E1/E2 heterodimer, anchored therein. Both proteins are strongly glycosylated and play a major role in the virus life cycle. They are involved in the process of the virus particle's entering into the host cell and in the process of depositing the infectious virus particles. Because of its RNA genetic material and no proof-reading activity of the RNA polymerase, the HCV demonstrates substantial genetic variability, which is the key factor enabling the virus to avoid the immune response. According to the most recent phylogenetic research, one can recognise seven genotypes and numerous subtypes of the virus, their variability reaching as high as 35%. Moreover, when infecting the organism, the virus diversifies forming closely related variants within a single organism infected. Furthermore, the E2 protein sequence has the so-called hypervariabie regions (HVR) which play the key role in the infection process. The virus's high genetic variability makes it extremely difftcuit to design new drugs, vaccines in particular. To make things worse, the virus has other strategies at hand to evade the host immune system (immune evasion), e.g. it impedes the intracellular signalling pathways responsible for the production of interferons, hinders activation of the antigen-presenting cells and the cell response, and moves directly from cell to cell without being exposed to contact with the host immune system. In addition, the giycans on the

particle surface enable the virus to mask its immunogenic regions (the glycan shield). The above strategies make designing an effective HCV vaccine an extremely tough challenge.

The ideal prophylactic vaccine should be geared against the strongly conserved epitopes accessible on the virus surface, which would induce both strong cell response from the T lymphocytes, and humoral response from the neutralising antibodies defending against the antigens of the E1 E2 glycoproteins of the HCV. Through the steric effect or through binding to the site involved in the interaction between the virus particle and the receptor of the host cell, the antibodies prevent virus penetration into the cell and the spreading of the infection. One of the epitopes for the neutralising antibodies is the strongly conserved QLINTNGSWHIN region located in position 412 to 423 of the E2 envelope glycoprotein. The region is recognisable to the broadly neutralising antibodies, capable to bind to the E2 glycoprotein of most of the HCV genotypes. The same site also plays the key role in the binding of the virus to the CD81 cell receptor which participates in viral penetration into the host cell. Moreover, the epitope is linear, which means that in order to enhance its antiviral immunogenicity it can be used as the peptide antigen of the surface carriers, i.e. virus-like particles proposed in this invention.

Virus-like particles (VLPs) are small, highly immunogenic structures formed of multimers of the viral proteins. Thanks to their multimeric, spatial structure, virus-like particles trigger aggregation of the BCRs (B cell receptors) on the B surface of the lymphocytes, and in effect activate humoral response of the organism. Furthermore, they are capable of stimulating response from T lymphocytes, specifically from the helper, cytotoxic, and dendritic cells. In view of their strong immunogenic properties, they are used in new generations of vaccines, e.g. in the vaccine against the Human Papilloma Virus (HPV) available in Poland since 2007, or the vaccine against the Hepatitis B Virus (HBV).

The genetic material of the Hepatitis B Virus is formed of a partially double-strand DNA which codes the virus's structural and functional proteins in partially overlapping reading frames. The HBV virion is ca. 42 nm in diameter, and contains the C protein core. The core is shielded in a protein-lipid envelope formed by the surface antigen of the HBV virus - HbsAg; the latter occurs in three forms: long, medium, and short. The short form of the protein - sHBsAg, where the amino acids are ca. 226 long, demonstrates interesting properties of creating virus-like particles. Those small structures, ca. 22 nm in diameter, are not virulent, but highly immunogenic and capable

of triggering strong immune response. The tertiary structure of the sHBsAg forms hydrophilic loops. On one of the loops, there is a strongly conserved determinant 'a' stimulating the strongest immune response. Due to its ability to form strongly immunogenic VLP particles, and its strongly conserved and exposed determinant 'a', the sHBsAg protein is used in the available anti-HBV vaccines. The vaccines are designed under the DNA recombination technique and produced in the yeast expression system. The system allows for obtaining the correctly folded sHBsAg protein in large volumes at low cost. The protein can be expressed in many other systems, mammalian included, where, unlike in the yeast system, it is glycosylated correctly and secreted to the medium. Unfortunately, protein expression in mammalian cells involves high production costs.

Due to its strong immunogenicity and capability of triggering strong cell and humoral responses, determinant 'a' of the HBsAg protein is frequently employed as the potential carrier of the human pathogen antigens. The research of recent years has studied the potential vaccines based on the sHBsAg protein which carries the antigens of e.g. the polio virus, human immunodeficiency virus (HIV), malaria parasite, and chikungunya virus. Furthermore, the description of invention EA 005313 B discloses a method of obtaining multivalent antigens by chemical combination of peptides into the so-called dendritic structures, following which the structures are chemically linked to the epitope carriers, e.g. the HBsAg protein.

The description of another invention, US 8.551.484 A, discloses neutralising antibodies of a broad spectrum of action, which interact with region 412 - 423 of the E2 glycoprotein of the HCV.

In the study below, with the view of obtaining a bivalent vaccine against the HBV and HCV chimeric protein was designed, where region 412-425 of the E2 envelope glycoprotein of HCV was inserted in determinant 'a' of the HBsAg protein originating from HBV, subtype adw2. Expression of the chimeric protein was obtained in a system based on a parasitic protozoa, the *Leishmania tarentolae. L tarentolae* is a unicellular, eukaryotic organism infectious to geckos but demonstrating no pathogenicity to humans. The protein expression system based on *L tarentolae* allows for obtaining high expression of correctly folded proteins with the N-glycosylation pattern, similar to that of the mammalian cells, though with the costs kept low, the handling easy, and posing no difficulty with increasing the culture scale, comparable to the ease of the yeast or bacterial cultures.

The purpose of the invention is to form virus-like particles and in effect create a bivalent vaccine against the HBV and HCV.

As such, the invention offers e.g. the following several advantages:

- triggering the immune response in the form of antibodies against HBV and/or HCV

- obtaining a bivalent vaccine designated for prophylaxis and/or treatment of HBV and HCV, which when administered protects from and/or suppresses the morbidity caused by HBV and/or HCV

- attaining the potential application of the vaccine combination for the purposes of prevention/prophylaxis and/or treatment of the HBV and/or HCV infections.

- obtaining biologically active and immunogenic virus-tike particles composed of the small envelope protein of the HBV (HBsAg), characterised by:

- high immunogenicity
- properties triggering humoral and cellular immune responses

- incorporating region 412-425 of the E2 glycoprotein of the HCV into determinant 'a', characterised by:

- low variability among the HCV genotypes
- presence of a binding site for the virus neutralising antibodies
- linear nature

- attaining the correct exposition of the E2 412-425 region on the surface of the HBsAg protein and its accessibility to the neutralising antibodies

- obtaining virus-like particles for the first time in the *Leishmania tarentolae* system the advantages of which are the following:

- correct protein folding
- presence of post translational modifications (e.g. N-glycosylation, where the giycosylation pattern is similar to that of the mammalian pattern)
- high overexpression of proteins
- low cost of the culture and its easy handling
- possible suspension culture, which enables problem-free increase of the production scale

- ensuring easy process of purifying the HBsAg_41 2-425 particles, consisting in single-stage gradient ultracentrifugation

The invention concerns a vaccine containing determinant 'a' of the HBsAg protein of the HBV, subtype adw2, and region 412-425 of the E2 envelope glycoprotein of the HCV.

The vaccine, where the sequence of determinant 'a' is as follows:

5'-TGCACAACTCCTGCTCAAGGAACCTCTATGTTTCCCT

CATGTTGCTGTAC AAAACCTAC GGACGGAAACTG CAC-3'

The vaccine, where the sequence of region 412-425 of the glycoprotein is as follows:

5'-CAACTG ATCAACACC AAC GGCAGTTG GCACATCAATAG CACG-3'

The vaccine is chimeric protein.

The vaccine which can be capable of triggering the patient's immune response.

The isolated chimeric protein, in the form of the vaccine defined above, is administered against viruses HBV and/or HCV,

The combination containing the vaccine being the chimeric protein defined above, appended with the instruction of its administration in prophylaxis of the HBV and/or HCV viral infections.

The method of obtaining the vaccine defined above, consisting of the following stages:

- insertion of the below-given sequence of region 412-425 of the E2 envelope glycoprotein of the HCV in the position corresponding to determinant 'a' of the HBsAg protein of the HBV virus, subtype adw2,

5'-CAACTGATCAACACCAACGGCAGTTGGCACATCAATAGCACG-3 '

- cloning of the synthesised fragment into plasmid pLEXSY_l-blecherry3 using restriction sites Bgl!l and Notl under the control of promoter T7 with tetracycline operator,

- the use of the prepared plasmid in electroporation of the *Leishmania tarentolae* cells,

- expression of protein HBsAg_41 2-425 in the *Leishmania tarentolae* cells, cell lysis, and particle formation,

- purification of the virus-like partic!es in single-stage gradient ultracentrifugation.

The composition intended for prophylaxis of HCV and/or HBV infections in a patient, where the composition contains the chimeric protein defined above, in a pharmaceutically acceptable carrier.

The expression vector being plasmid pLEXSYJ_blecherry3 containing the nucleotide sequence of the chimeric gene HBsAg_412-425 and restriction sites Bglll, Notl, presented on Fig. 2.

The following terms are defined for the purposes of the present invention:

HCV - denotes Hepatitis C Virus, Hepatitis C.

HBV - denotes Hepatitis B Virus, Hepatitis B.

antigen - denotes any substance which stimulates the organism's immune system to produce antibodies geared against the antigen.

fusion protein - is the same as chimeric protein.

bivalent vaccine - denotes the vaccine which triggers immune response against two different pathogens (or two different pathogen serotypes). In this particular case, it denotes the vaccine which triggers immune response against both the HBV and/or the HCV viruses.

chimeric proteins - denote the proteins produced in effect of expression of recombinant genes obtained in combination of two or more genes, each coding different proteins originally. In this particular case, they denote the combination of the short form of the HBV virus surface antigen (HBsAg) and region 412-425 of the E2 glycoprotein of the HCV, where the E2 412-425 region is inside determinant 'a' of the HBsAg.

determinant 'a' - denotes the highly immunogenic region of the small envelope protein HBsAg, located in position 124-147 of the small envelope protein of the HBV virus - sHBsAg (Genbank accession number - AF397207.1).

expression - denotes production of proteins in effect of translation of the protein coding genes.

hypervariable region - denotes the regions in the amino acid sequence of glycoprotein E2, which demonstrate increased variability. Variability is the key factor enabling the HCV particle to evade the immune response.

cellular response - denotes the specific immune response of the organism, involving the occurrence of T lymphocytes with receptors on their surface. The receptor's binding to a specific antigen triggers direct, cytotoxic response from the T lymphocyte, or activation of other effector cells.

humoral response - denotes the specific immune response of the organism, involving the production of antibodies by the activated B lymphocytes.

epitope, antigen determinant - denotes the site on the antigen which specifically binds the antibody/immunoglobulin (e.g. the E2 412-423 region specifically binding the AP33 antibody). Among the epitopes one can distinguish linear epitopes - where the region binding the antibody is linear and does not depend on the protein tertiary structure, and the denaturation factors do not affect the ability of binding the antibody to the epitope, and conformational epitopes - where the binding of the antibody depends on epitope presentation in the protein tertiary structure.

virus neutralisation - denotes the ability of the neutralising antibodies to bind to the virus particle in such a way that the latter is unable to penetrate the host cell.

glycosylation pattern - denotes the pattern of the hydrocarbon particles covaiently bound to a protein particle.

region 412-425 - denotes the region in position 412 to 425 of the E2 glycoprotein of the HCV, isolate H77 (Genbank accession number – AF01 1751) of the QLINTNGSWHINST amino acid sequence. The region contains a strongly conserved linear epitope recognised by the neutralising antibodies of a broad spectrum of action, are able to bind to the E2 glycoprotein originating from most HCV genotypes. In this particular case, region 412-425 was used to form chimeric protein HBsAg_412-425, where the region is inside determinant 'a' of the HBsAg protein.

peptide 412-425 - denotes the synthesised peptide of the *QLINTNGSWHINST* amino acid sequence.

antibody AP33 - denotes the mouse monoclonal antibody of the QLINTNGSWHIN sequence, binding to the linear epitope of the E2 glycoprotein of the HCV. The antibody demonstrates properties neutralising the HCV. (A.Owsianka.A.W. Tarr,V. S. Juttla et al., "Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein," Journal of Virology, vol. 79, no.17, pp. 11095-11104, 2005).

HBV virus, subtype adw - denotes the variant of the HBV found to have determinants 'a', 'd', and 'w'. The HBV virus has three determinants, out of which determinant 'a' is universal and common for all subtypes. The flanking regions of determinant 'a' are called determinants d/y and w/r. Thanks to the type of the amino acid in position 122 for determinant d/y, and in position 160 for determinant w/r, each isolate of the HBV virus can be ascribed to a specific subtype.

patient - denotes a person uninfected with HBV and/or HCV, who is vaccinated with chimeric protein HBsAg_412-425 to prevent HBV and/or HCV infection.

infection prophylaxis - denotes prevention of new HBV and/or HCV infections by administering prophylactic vaccinations of the individuals exposed to the HBV and/or HCV infections with chimeric protein HBsAg_412-425. The vaccination triggers permanent immune response in the form of memory cells which, if in contact with the HBV and/or HCV viruses, are able to neutralise and eliminate the HBV and/or HCV viruses from the organism of the vaccinated person.

Description of the figures:

Fig. 1 - presents a diagram of the chimeric HBsAg_412-425 construct used in the study below. Region 412-425 originating from the E2 envelope glycoprotein of the HCV was inserted in position P127/A128 of the small envelope protein of the HBV virus (HBsAg), within determinant 'a'.

Fig. 2 - presents a diagram of the plasmid pLEXSY_I-blecherry3 with the fragment coding protein HBsAg_412-425 cloned in under the control of the tetracycline promoter.

Fig. 3 - presents the fractions containing protein HBsAg_412-425 upon ultracentrifugation, separated on SDS-PAGE gel under reducing conditions after their

staining with Coomassie Blue. The arrow on the figure points to the monomeric form of protein HBsAg_412-425 with the molecular weight of t ~27 kDa. The kDa molecular weight marker is positioned left of the gel.

Fig. 4 - presents the result of the ELISA test, confirming a positive response from the antibodies to the chimeric HBsAg_412-425 particles. The plates were coated with *L. tarentolae* lysate containing chimeric protein HBsAg_412-425 in dilutions of the following ranges: 1, 1:10, 1:100, 1:1000, 1:10000. Subsequently, incubation with the AP33 antibodies (Fig. 4A) and anti-HBsAg (Fig. 4B) was conducted. The error bars mark the standard deviation.

Fig. 5 - presents a photograph of the preparation of the virus-like HBsAg_421-425 particles viewed under an electron microscope. In the experiment, the technique of negative staining with uranyl acetate was employed. The black arrow points to a single virus-like particle composed of chimeric protein HBsAg_412-425.

Fig. 6 - A - presents titre determination of the antibodies in the serum of mice immunised with protein HBsAg_412-425. 10 Mg/ml of purified chimeric virus-like particles was applied to a 96 well plate. The protein was detected with sera in the following dilutions: 10^4, 10^5, $2x10^5$, $4x10^5$, $8x10^5$, $1.6x10^6$. **B** - 20 µg/ml of the earlier synthesised peptide 412-425 was applied to a 96 well plate. The peptide was detected with sera in the following dilutions: 10^2, 10^3, $1.25x10^3$, 10^4, 10^5. **C** - presents interaction of the immune sera with yeast-derived HBsAg proteins. The ELISA plates were coated with 5 µg/mL of purified HBsAg protein from *P. pastoris* (yHBsAg). The protein was detected with sera in the following dilutions: 10^3, $5x10^3$, 2.5x10", 1.25x10^5, 6.25x10^5. Shown is one of two representative experiments in duplicate. The mean A_{450} values and the standard deviations are shown on the y-axis. **D** - analysis of cross-reactivity of the HBsAg_412-425 sera to the E1E2 complex from different HCV genotypes. The figure represents western blotting in reducing conditions with HBsAg_412-425 sera diluted 1:500. As the antigen, extracts of the HEK293 cells transfected with plasmids expressing the E1E2 glycoproteins from different HCV genotypes were used. Non-transfected HEK293 cell lysate was used as the negative control (NC).

The invention is illustrated with the following exemplary, though not exhaustive, embodiments:

EXAMPLE 1

Obtaining expression of protein HBsAg_412-425 in the cells of the *Leishmania tarentolae* protozoa, and purifying the virus-like particles in gradient ultracentrifugation.

Expression

The gene coding protein HBsAg of the HBV virus was synthesised (Life Technologies Inc. USA) based on a database sequence (GenBank accesion number - AF397207.1), the sequence for region 412-425 - 5'-CAACTGATCAACACCAACGGCAGTTGGCACATCAATAGCACG-3' was inserted in

the position corresponding to determinant 'a' (Fig. 1). Then, the synthesised fragment was cloned into plasmid pLEXSY_i-blecherry3 (Jena Bioscience, Germany) using restriction sites BglII and NotI, under the control of promoter T7 with tetracycline operator. The correctness of the insert introduction was verified in sequencing. The thus prepared expression vector (Fig. 2) was used in the electroporation of the L *tarentolae* cells. The electroporation, polyclonal selection, cell culture, and induction of the expression of the chimeric protein were carried out in accordance with the procedure for the Lexsy system (Jena Bioscience, Germany).

Lysis and formation of virus-like particles

The induced cultures (OD600-4.5) were centrifuged (4500 rpm, 10 min). The cell pellets obtained from 100 ml of the culture were washed with the PBS buffer and then lysed in 10 ml of the buffer (0.6% Triton X-100, 0.6 mM reduced glutathione in PBS) and sonified. Then, the whole lysate was centrifuged (8500 rpm. 30 min.). After centrifugation, the supernatant was collected and incubated in room temperature for (12-48h).

Purification

In order to purify the HBsAg_41 2-425 particles, OpitPrep gradient (Sigma) was prepared in PBS with fractions ranging from 6% to 30%, onto which the obtained cell lysate was placed in 5 ml portions. The samples were centrifuged (28000 rpm, 4°C, 16h). All fractions formed on the gradient were tested for the presence of protein HBsAg_41 2-425 with the western blot method using the AP33 antibodies. The purity of the protein was tested by polyacrylamide gel electrophoresis, SDS-PAGE (Fig. 3). On the Coomassie Blue stained gel, one can see the main strip corresponding to the weight of the monomeric form of protein HBsAg_41 2-425 (27 kDa) plus 3 additional strips of higher weights, most likely corresponding to multimeric forms of protein HBsAg_41 2-425. The fractions containing the desired protein in largest quantities were collected and densified using Amicon 30 NMLW centrifugal filters (Millipore Inc. USA). The Bradford method was employed to determine protein concentration (A=595 nm). Estimate calculations revealed that the protein quantities stood at about 20 mg/l of the culture. The thus purified preparation was stored at 4°C or at -70°C with admixture of glycerol (5-20%).

The above procedures enabled obtaining of the chimeric protein and effective single-stage purification of the virus-like particles composed of fusion protein HBsAg_412-425.

EXAMPLE 2

The ELISA test conducted and electron microscope photographs taken to verify the presence of the virus-like particles.

ELISA

In order to perform the ELISA test, a 96-well plate was coated with cell lysate in PBS dilutions of 1 to 1:10000, and blocked with 5% milk in PBS-T (0.05% Tween20 in PBS). Then, applied were primary antibodies anti-HBsAg (1:1000) in PBST (Fig. 4B), or AP33 antibodies (1:1500) in PBST (Fig. 4A). In the experiment, HRP conjugated secondary antibodies were used at the dilution of 1:2000. The response was developed using the TMB substrate, and the result was read at the wavelength of 450 nm. The positive response of the antibodies to the chimeric HBsAg_412-425 particles confirms the correct exposure of region 412-425 on the surface of the HBsAg protein and its accessibility to the antibodies produced in contact with the antigen.

Electron microscopy

In order to verify the presence of the virus-like particles, 10 µl of the sample was applied to nickel grids coated with a carbon film with Formvar admixture and stained with uranyl acetate. The preparations were viewed in an electron microscope (Fig. 5). The findings of the electron microscopy verified the presence of virus-iike particles ~ 22 nm in diameter, the fact corresponding with the literature data.

EXAMPLE 3

Immunisation of mice to verify immunogenicity of the virus-like particles, and determination of the titre of the antibodies active against the HBsAg_412-425 antigen in the obtained sera

In order to test the immunogenicity of the virus-iike particles, the mouse model was employed in vaccinations. The purified HBsAg_412-425 antigen was suspended in PBS and mixed with the adjuvant (Addavax; Invivogen, USA) in the 1:1 proportion directly before the vaccination. A group of six BALB/c mice was inoculated subcutaneousiy with the antigen/adjuvant mixture at the dose of 15 pg/mouse. The control group of two BALB/c mice was inoculated with a PBS/adjuvant mixture. On the 14th and 28th day after the first inoculation, the mice were administered the antigen in a lower quantity, i.e. at the dose of 10 pg/mouse, whereas the control group was administered the PBS/adjuvant mixture. On the 42nd day, all mice were bled and the collected serum subject to determination of the titre of the antibodies active against the whole antigen HBsAg_412-425 and region 412-425 in addition. The titre was

determined under the EL1SA method (Fig. 6A and B). The above experiment confirmed the presence of antibodies active against protein HBsAg_412-425, the titre of which was about $8x10^5$; it also confirmed the presence of antibodies active against region 412-425, the titre of which was about $1.25x10^3$. In both cases the negative result was assumed to represent values (n<0.15), where 0.15 is three times the value of the negative control (A_{450}=0.05). The immunisation results confirm a strong immune response in the form of antibodies active against the antigen given the form of chimeric protein HBsAg_412-425, including region 412-425 specific for the HCV. Additionally, detailed serum characterisation revealed cross-reactivity with purified yeast-derived HBsAg protein (yHBsAg) (Fig. 6C). The result indicated that the sera contained high titres of anti-HBsAg antibodies, suggesting that 14 aa foreign epitope insertion within a sHBsAg particle does not interfere dramatically with the humoral response against the sHBsAg protein itself. Furthermore, the tested sera were also able to cross-react with the E1E2 complexes from different HCV genotypes. As expected, the sera recognised the E2 glycoproteins from HCV genotypes 1a, 1b, 2b, 3a, 4, and 6, but failed to recognise the E2 derived from genotype 5 (Fig. 6D). That was due to the fact that the isolate had 4 amino acid changes in region 412-423 unlike the sequence used to create the HBsAg_412-425 protein. Considering the above, the 412-425_HBsAg sera showed broad cross-reactivity across various HCV genotypes.

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Claims

1. A vaccine containing determinant 'a' of the HBsAg protein of the HBV virus, subtype adw2, and region 412-425 of the E2 envelope glycoprotein of the HCV.
2. The vaccine according to Ciaim 1, **characterised in that** the sequence of determinant 'a' is as follows:

   S'-TGCACAACTCCTGCTCAAGGAACCTCTATGTTTCCCT

   CATGTTGCTGTACAAAACCTACGGACGGAAACTGCAC-3'

3. The vaccine according to Claims 1-2, **characterised in that** the sequence of the E2 glycoprotein 412-425 region is as follows:

   5'-CAACTGATCAACACCAACGGCAGTTGGCACATCAATAGCACG-3'

4. The vaccine according to Claims 1-3, **characterised in that** the vaccine is chimeric protein.
5. The vaccine according to Claims 1-4, **characterised in that** it can trigger the patient's immune response.
6. The isolated chimeric protein in the form of the vaccine defined in Claims 1-5 is administered against viruses HBV and/or HCV.
7. A combination containing the vaccine being the chimeric protein defined in Claims 1-5, appended with the instruction of its administration in prophylaxis of the HBV and/or HCV viral infections.
8. A method of obtaining the vaccine defined in Claims 1-5, consisting of the following stages:

   - insertion of the beiow-given sequence of region 412-415 of the E2 envelope glycoprotein of the HCV in the position corresponding to determinant 'a' of the HBsAg protein of the HBV, subtype adw2

   5'-CAACTGATCAACACCAACGGCAGUGGCACATCAATAGCACG-3'

   - cloning of the synthesised fragment into plasmid pLEXSYJ-blecherry3 using restriction sites Bglll and Not! under the control of promoter T7 with tetracycline operator,

- the use of the prepared plasmid in electroporation of the *Leishmania tarentolae* ceils,

- expression of protein HBsAg_41 2-425 in the *Leishmania tarentolae* cells, cell lysis, and particle formation,

- purification of the virus-like particles in single-stage gradient uitracentrifugation.

9. A composition intended for prophylaxis of HCV and/or HBV infections in a patient, **characterised in that** the composition contains the chimeric protein defined in Claims 1-4, in a pharmaceutically acceptable carrier.

10. An expression vector **characterised in that** it is plasmid pLEXSY_I_blecherry3 containing the nucleotide sequence of the chimeric gene HBsAg_41 2-425 and restriction sites Bg!ll, Notl, presented on Fig. 2.


sHBsAg
1
„a" determinant
226
124 CTTPAQGTSMFPSCCCTKPTDGNC 147
HCV E2 glycoprotein
384 HVR1
TMD
746
412 QLINTNGSWHINST 425
HBsAg_412-425
1
240
124 CTTPQLINTNGSWHINSTAQGTSMFPSCCCTKPTDGNC 161

Fig. 1

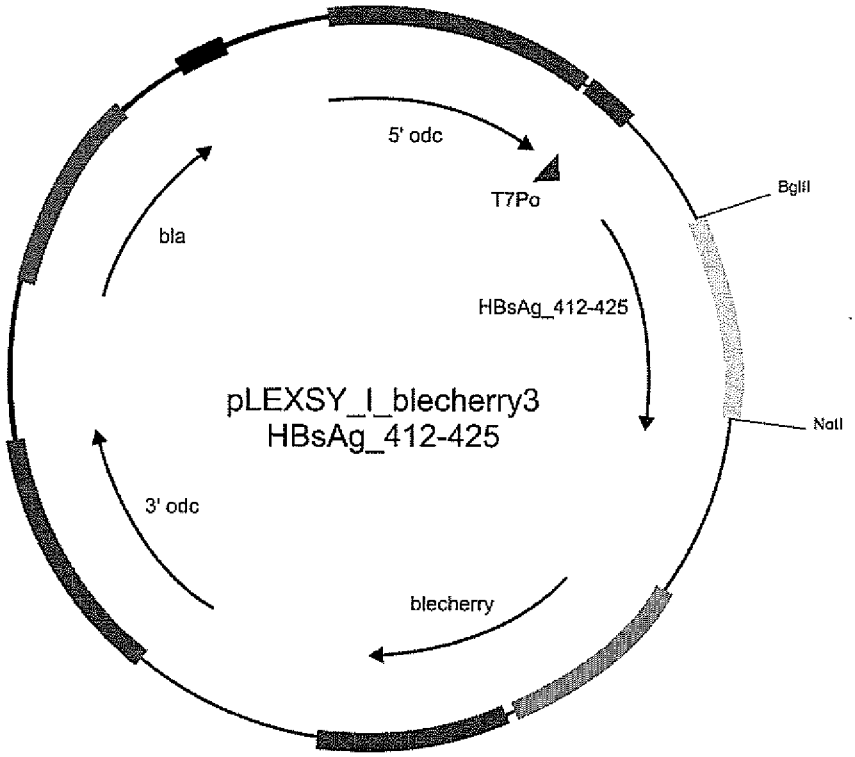
5' odc
T7Po
BglII
bla
HBsAg_412-425
pLEXSY_I_blecherry3
HBsAg_412-425
NotI
3' odc
blecherry

Fig. 2

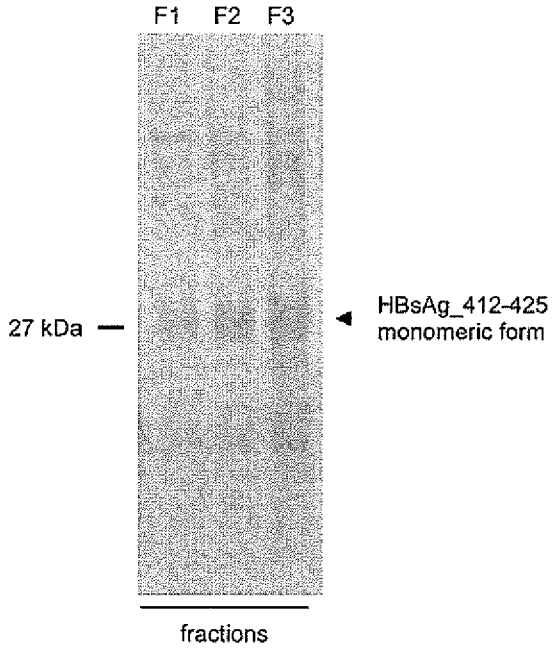
F1
F2
F3
27 kDa
HBsAg_412-425
monomeric form
fractions

Fig. 3

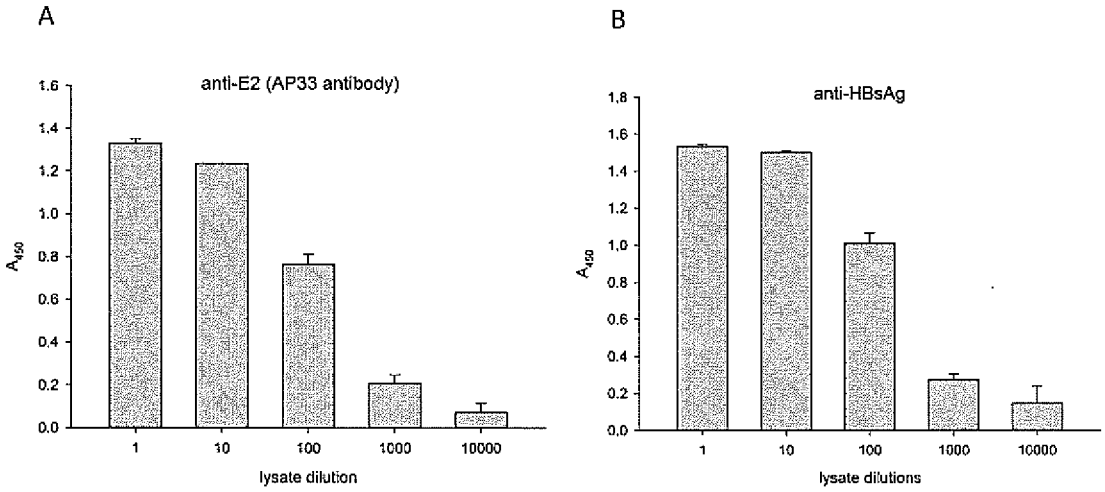
A
anti-E2 (AP33 antibody)
A_{450}
lysate dilution
B
anti-HBsAg
A_{450}
lysate dilutions

Fig. 4

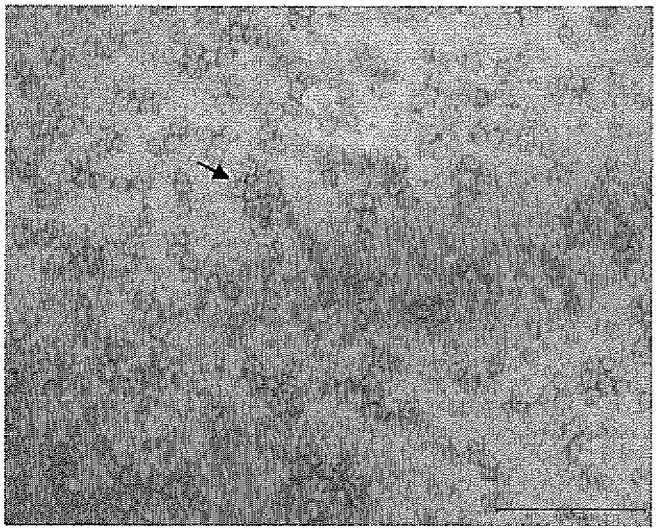

Fig. 5

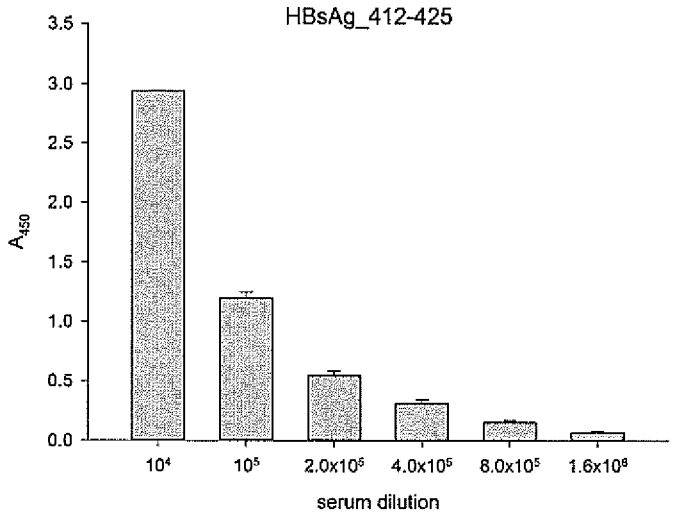
HBsAg_412-425
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A450
10⁴
10⁵
2.0x10⁵
4.0x10⁵
8.0x10⁵
1.6x10⁶
serum dilution

Fig. 6A

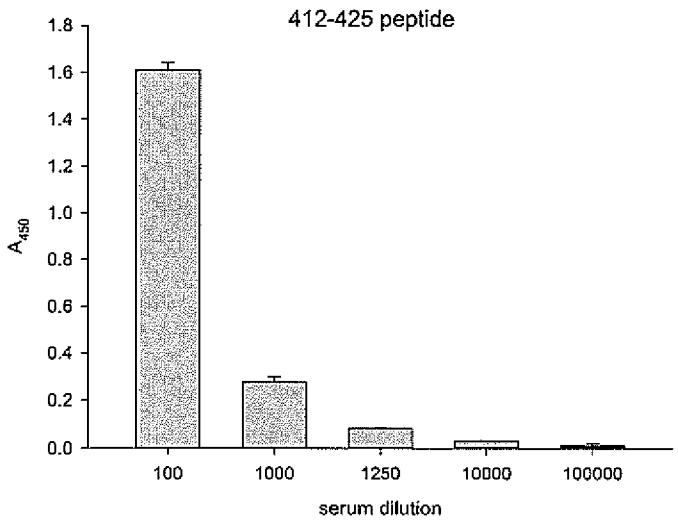

Fig. 6B

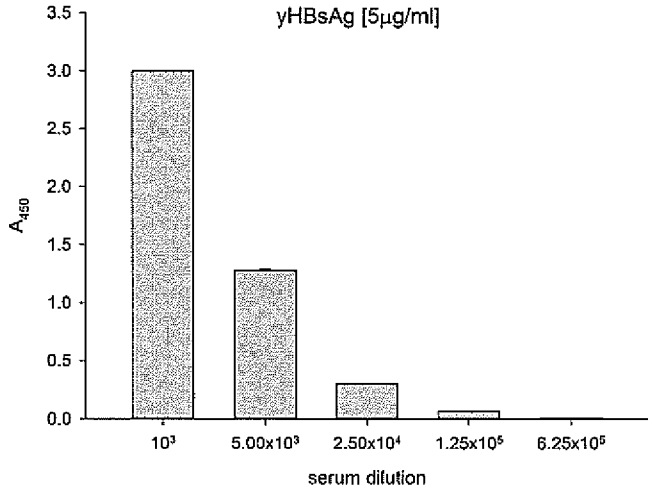

Fig. 6C

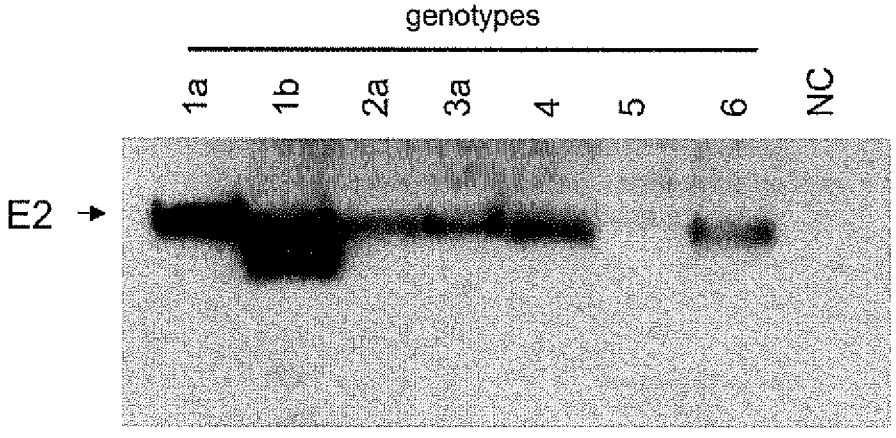

Fig. 6D

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2016/0O0O02

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/29 C12N15/85 A61K39/O0
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/10416 Al (CROWN IN THE RIGHT OF THE QUEE [AU] ; GOWANS ERIC JAMES [AU] ; MACNAUGHT) 7 February 2002 (2002-02-07)	1-7,9
Y	figure IB page 2, line 22 - line 26	8, 10

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 May 2016	17/05/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lanzrei n, Markus

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2016/000002

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VIETHEER PATRICIA T K ET AL: "Immunizations with chimeric hepatitis B virus-like particles to induce potential anti-hepatitis C virus neutralizing antibodies" , ANTIVIRAL THERAPY- AN OFFICIAL PUBLICATION OF THE INTERNATIONAL SOCIETY FOR ANTIVIRAL RESEARCH , MTM PUBLICATIONS, LONDON, GB, vol . 12, no. 4, 1 January 2007 (2007-01-01) , pages 477-487, XP009180161 , ISSN: 1359-6535 page 480, left-hand column, paragraph 3; table 1	1-10
A	WO 2008/108918 A1 (UNIV MASSACHUSETTS [US] ; BROERING TERESA [US] ; SLOAN SUSAN [US] ; BABCO) 12 September 2008 (2008-09-12) page 23, line 30 - line 33 page 24, line 5	1-10
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T	ANNA CZARNOTA ET AL: "Immunogenicity of Leishmania-derived hepatitis B small surface antigen particles exposing highly conserved E2 epitope of hepatitis C virus" , MICROBIAL CELL FACTORIES, vol . 351 , no. 27 , 13 April 2016 (2016-04-13) , page 2832 , XP055270806, DOI: 10. 1186/S12934-016-0460-4	

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Information on patent family members

International application No
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		DK 2125889 T3	14--04--2014
		EP 2125889 Al	02-12--2009
		ES 2456961 T3	24--04--2014
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		RS 53258 B	29--08--2014
		SI 2125889 Tl	30--06--2014
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		wo 2008108918 Al	12--09--2008
W0 2009061739 **Al**	14-05-2009	US 2010247522 **Al**	30-09-2010
		W0 2009061739 **Al**	14-05-2009