IN THE APPLICATION

OF

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FOR

AN INNOVATIVE VACCINE AGAINST HEPATITIS C VIRUS

SPECIFICATION

TITLE OF THE INVENTION

AN INNOVATIVE VACCINE AGAINST HEPATITIS C VIRUS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to the Hepatitis C vaccine, where the vaccine against Hepatitis C Virus (HCV) was developed using Bovine Viral Diarrhea Virus (BVDV) NS3/NS4a for possible prevention of HCV 1a and 1b in human. This invention is related to various fields such as virology, pathology, immunology and molecular biology.

DESCRIPTION OF THE PRIOR ART

NS3/NS4a protease is essential for Hepatitis C Virus (HCV) and Bovine Viral Diarrhea virus (BVDV) and Flaviviruses replication, and is one of the most promising targets for specific anti-HCV therapy and vaccine development. The protease NS3/ NS4a genes of Flaviviruses are considered to be one of the less variable genes among HCV and BVDV genomes. In the present invention, the genetic heterogeneity of the NS3/NS4 protease gene was analyzed in HCV genotypes and subtypes samples collected from HCV infected

patients and BVDV infected cattle. The NS3/4A sequence was amplified from the sera of an HCV 1a and 1b genotype-infected patients and BVDV infected cattle using the Polymerase Chain Reaction (PCR). Total RNA was extracted from serum, and cDNA synthesis and PCR were performed according to standard protocols. A total of 284 isolates of HCV and 197 of BVDV isolates were sequenced. Variability in one or more nucleotide and amino acid sequences was found between 1a and 1b subtypes from patients and among BVDV isolated from infected cattle. However, most non-structural regions were conservative. This substantial polymorphism of the NS3/NS4a protease produced by HCV-1a and HCV-1b and BVDV isolates suggests that, despite the numerous functional and nucleotide structural constraints, the enzyme is sufficiently flexible to tolerate substitutions among these same family viruses. That triggers our invention to develop vaccine against HCV using BVDV NS3/NS4a for possible prevention of HCV 1a and 1b in human.

The BVDV antigen library comprising nucleic acids that encode the most conserved multivalent antigenic polypeptide inducing an immune response against Hepatitis C genotype-1a and b.

Screening the library of Hepatitis C viruses -1a and b, Bovine Viral Diarrhea Viruses for conserved nucleic acid sequences (NS3/ NS4a) that encode at least one antigenic polypeptide comprising multiple homologous sequences, each viral sequence being positioned relatively close respective antigenic polypeptide.

Selection of Nucleic acid antigens of Flaviviruses : Expression vector and bacteria for N3/NS4a : The method of the screening of nucleic acid from Bovine Viral Diarrhea Virus cDNA encodes for NS3/NS4a were cloned into pGx2T vector, at BamH1 site for expression as a fusion protein in JM109 E. coli. The plasmid vector contains the glutathione-S-transferase (GST) gene under the control of TAC promoter. A recombinant bacterium carrying gene for NS3/NS4a was multiplied in LB broth, and the bacterial pellet was collected by centrifugation at 4000 X g for 10 min. The polypeptides were isolated from the bacterial pellet, denatured by urea, and renatured with dialysis in PBS. The amount of protein was determined by a BioRad DC protein assay kit. The purified polypeptides were analyzed by SDS-PAGE followed by immunoblotting. The polypeptides transferred onto nitrocellulose paper were probed with a 1:100 dilution of human HCV-positive sera and negative sera obtained from 87 patients either infected with HCV 1a (67) or 1b (20). Then affinity-purified peroxidase-labeled goat anti-bovine IgG (H+L) (KPL Inc. Galthersburg, MD) was added, and the nitrocellulose papers were developed for color reaction in 4-Chloro-1-naphthol with 30% H_2O_2.

Purification of recombinant fusing proteins using a glutathione sepharose column: As described earlier, the pGex-2T vector containing the GST gene were under the control of TAC-promoter. The GS 4B column (Pharamacia Biotech, NJ) consists of glutathione coupled via 10-carbon spacer arms to the oxirane group of epoxy-activated sepharose B. The gel is designed for the purification of recombinant fusion proteins containing the carboxyl terminus of GST as an affinity tail. The GS 4B column was used to isolate the recombinant fusion proteins from E. coli. Pharmacia protocol and buffers were used to

trap and elute fusion proteins. Elution of fusion proteins from columns were determined by monitoring with an ISCO UA-5 UV fraction detector (ISCO Inc., Lincoln, NE). The eluted fusion proteins were run on SDS-PAGE and stained with Commassie blue. The quantity of protein was estimated by using a Bio-Rad DC protein assay kit.

Animal studies: Balb/C mice were immunized with r-NS3/NS4 protein to induce humoral and cell mediated immune responsiveness against HCV 1 and 1b is accomplished by introducing into a test animal either: a) One nucleic acid encoding NS3/NS4a of the BVDV viruses and in combination of nucleic acid of the BVDV virus library of nucleic acids to determine induction of immune responses against each of the HCV genotype 1a and b, which polypeptides that is greater than the immune response induced by any one and antigenic polypeptides pool against any other of the BVDV conserved antigenic polypeptides.

Further, the library is subdivided into groups, each of which group is introduced into a test animal to screen for groups that include at least one BVDV nucleic acid that encodes at least one BVDV antigenic polypeptide encodes for NS3/NS4a that produce sensible immune response against each genotype of HCV 1a and b.

Wherein the immunization of animals is accomplished by: Expressing each recombinant protein of the library of nucleic acids in bacteria expression vectors such that at least one antigenic polypeptide encoded by a nucleic acid of the library.

Immune responses: The antigen library of BVDV, wherein at least one viral nucleic acid and one recombinant multivalent antigenic polypeptide encodes that induces immune response to the genotypes of HCV 1a and b. Wherein antigenic polypeptides are different strains of BVD viruses belongs to Cytopathic and non-Cytopathic species, and at least one nucleic acid encodes at least one antigenic polypeptide that induces immune response to genotypes of HCV 1a and b.

Wherein at least one nucleic acid in the library encodes at least one antigenic polypeptide that induces an immune response against each of the HCV genotype that is greater than the immune induced by the other nucleic acid of a BVD virus.

Wherein antigenic polypeptides are different genotypes, serotypes, strains, or species of BVDV, and combination of nucleic acid encodes antigenic polypeptides that induces immune response to each of the of the genotype of HCV 1a and b.

The antigen library of multivalent antigenic polypeptide further comprises multiple non-contiguous subsequences of a fourth antigenic polypeptides of BVDV induce immune responses to the genotype of HCV 1a and b. Antigenic polypeptide of a BVDV immunized animals with at least one nucleic acid in the library encodes at least one antigenic polypeptide or in combination of polypeptides of that induces an immune response against each of the HCV genotype-1a and/or HCV genotype-1b that could exhibit a high degree of antigen induced proliferations responses of spleen and T-cells with significance difference between HCV genotypes compare to controls.

SUMMARY OF THE INVENTION

Worldwide, more than one million new cases of HCV infection are reported annually. In the United States alone, nearly four million persons are infected and 30,000 acute new infections are estimated to occur annually. Currently, HCV is responsible for an estimated 8,000 to 10,000 deaths annually in the United States, and without effective intervention, that number is predicted to triple in the next 10 to 20 years. Furthermore, HCV is the leading reason for liver transplantation in the United States and this has major implications in the present era of organ shortage. The ultimate goal is a universally effective vaccine to prevent new cases, especially in underdeveloped countries, where HCV infection is more prevalent and treatment is financially out of reach for most patients. The development of vaccine has been hampered, at least partly, by the great heterogeneity of the HCV genome, which is the focus of this claim. On the basis of phylogenetic analysis of nucleotide sequences, multiple genotypes and subtypes of hepatitis C virus (HCV) have been identified. Characterization of these genetic groups is likely to facilitate and contribute to the development of an effective vaccine against infection with HCV. Differences among HCV genotypes in geographic distributions have provided investigators with an epidemiologic marker that can be used to trace the source of HCV infection in a given population. HCV genotype 1 may represent a more aggressive strain and one that is less likely to respond to interferon treatment than HCV genotype 2 or 3. However, these observations require confirmation before HCV genotyping can be used in clinical settings. HCV types 4 through 9, have been found

mostly in less industrialized countries (India and countries in Southeast Asia and the Middle East).

At least six major genotypes of HCV, each comprising multiple subtypes, have been identified worldwide. Substantial regional differences appear to exist in the distribution of HCV genotypes Although HCV genotypes 1, 2, and 3 appear to have a worldwide. HCV subtypes 1a and 1b are the most common genotypes in the United States. These subtypes also are predominant in Europe. In Japan, subtype 1b is responsible for up to 73% of cases of HCV infection. Although HCV subtypes 2a and 2b are relatively common in North America, Europe, and Japan, subtype 2c is found commonly in northern Italy. HCV genotype 3a is particularly prevalent in intravenous drug abusers in Europe and the United States. HCV genotype 4 appears to be prevalent in North Africa and the Middle East, and genotypes 5 and 6 seem to be confined to South Africa and Hong Kong, respectively. HCV genotypes 7, 8, and 9 have been identified only in Vietnamese patients, and genotypes 10 and 11 were identified in patients from Indonesia. There has been disagreement about the number of genotypes into which HCV isolates should be classified. Investigators have proposed that genotypes 7 through 11 should be regarded as variants of the same group and classified as a single genotype, type 6.

HCV was a positive-sense RNA virus with approximately 9,400 ribonucleotides, containing a poly (A) tail at the 3' end. The sequence contained a 5' untranslated region (5' UTR) of 341 bases, a long open reading frame coding for a polyprotein of 3,011 amino acids, and a 3' untranslated region (3' UTR) of about 27 bases. This RNA structure is most similar to that of the family Flaviviridae, which encompasses numerous arthropod-

borne viruses. Consistent with the known functions of most Flaviviruses proteins, the three N-terminal HCV proteins are probably structural (C, E1, and E2/NS2) and the four C-terminal proteins (NS2, NS3, NS4, and NS5) are believed to function in viral replication.

Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of cattle with a worldwide distribution. It is classified as a Pestivirus within the Flaviviridae family and is divided into genotypes 1 and 2. In addition, 11 BVDV subtypes of genotype and 2 subtypes of genotype 2 have been described. BVDV are nonsegmented, single, sense stranded (positive polarity (+) RNA viruses, now classified as a genus of the family Flaviviridae. The Flavivirus family has 3 genera: Flaviviruses, Pestiviruses, and Hepatitis C-like viruses. The genomes of BVDV consist of 1 long open reading frame (ORF), flanked by 2 untranslated regions (UTR). The ORF is translated into 1 long polypeptide, which is subsequently cleaved into the individual viral proteins by viral and cellular proteases. With the exception of the first protein, Npro, which is one such viral protease, the BVDV genome is organized with the structural protein genes (the capsid (C), and 3 envelope glycoproteins) at the 5' end of the ORF and the nonstructual protein genes (NS) occupying the last two thirds of the ORF. There are 6 nonstructural protein genes on the noncytopathic BVDV genome (NS2/3, 4A, 4B, 5A, and 5B). The nonstructural proteins are either known or assumed to be involved with virus replication. The BVDV gene names and numbers have been designated as such to coincide with the genes of other Pestiviruses and the Flaviviruses, and the Hepatitis C-like viruses, which share a similar genomic arrangement.

Quasispecies genotypes, and other subspecies categorizations have been described in vesicular stomatitis virus, Hepatitis C virus (HCV), and BVDV. The study of quasispecies has shown that, despite the stochastic processes of replication and mutation that lead to RNA virus diversity, the population dynamics of RNA virus quasispecies is a nonlinear, deterministic evolutionary behavior. All the factors involved in such selection are numerable and unknown, but most certainly include host factors.

Competition among quasispecies is evident in chronic HCV infections. Individuals infected with HCV can be superinfected with different strains, leading to eradication or suppression of the original infecting strain. The ultimate outcome of multiple HCV exposures may be dependent on a single strain establishing its dominance. Another HCV quasispecies phenomenon that has been reported is the ability to change quasispecies populations in response to homologous rechallenge. The reincocualtion of chimpanzees that were chronically infected with HCV with the same HCV strain resulted in the emergence of minor quasispecies and a shift in the major quasispecies population. More interesting is that this shift in quasispecies dynamics resulted in clinical changes in serum alanine aminotransferase levels, viral load, and antibody titers all rose. Hepatocellular ultrastructural changes that typify HCV infection, which had disappeared prior to reinfection, reappeared.

Environmental factors also affect quasispecies populations. Studies have shown that a minor quasispecies population in humans with chronic HCV infections became the predominant quasispecies during interferon therapy. Quasispecies variation also occurs in Pestiviruses. Although there are no published studies on competition among Pestivirus

quasispecies, it is likely that the same type of population dynamics exists among quasispecies of Pestivirus as among those of HCV. The E2 region in BVDV also contains a hypervariable region or genomic “mutational hot spot” which can be expected to produce quasispecies variation. It is this protein that elicits BVDV neutralizing antibodies. However, antibodies to a different, more conserved region of this same protein can be used to determine BVDV genotypes. Originally BVDV was segregated into 2 genotypes (BVDV1 and BVDV2), based on the 5' untranslated region (UTR). This region does not code for a protein; however, it is important for the initiation of translation of the RNA ORF and is highly conserved. Subsequently, other regions of the BVDV genome were also found to be highly conserved and, when sequenced, will generally group viruses in much the same way as does the 5'UTR. Based on these conserved regions, phylogenic analysis suggested that BVDV and HCV are not as different from each other. This suggests that BVDV genomes are as not distinctly different as genomes of HCV. As compelling as this seems, such genotypic comparisons are based on arbitrarily chosen genomic regions, which may not code for viral proteins and are of uncertain significance in terms of human and animal disease.

The HCV replicase is cytoplasmic and membrane associated, consisting of at least NS3-NS5B, together with additional as yet undefined host components. The major enzymatic components of the RNA replication machinery are the NTPase/helicase activity located in the C-terminal two-thirds of NS3 and the NS5B RNA-dependent RNA polymerase (RdRP). Both of these enzymes have been extensively studied biochemically and have yielded high-resolution structures that are aiding drug discovery efforts. The first step in

the RNA replication process involves the synthesis of a complementary negative-strand copy of the incoming genome RNA.

Efficient assembly and release of HCV particles has not been recapitulated in the laboratory. Thus, whereas these are attractive steps for intervention, it has not been possible to establish systems to study them or for antiviral screening. Based on results with other members of the Flaviviridae, it seems likely that HCV buds into intracellular vesicles and is transported out of the cell by the host secretory pathway and released into the extracellular space. It is interesting that iminosugars, that act as glucosidase inhibitors to interfere with proper carbohydrate maturation of glycoproteins, have been shown to inhibit infectious virus production of a another member of this family, the Pestivirus bovine viral diarrhea virus (BVDV) for a recent review of the antiviral potential of iminosugars.

NS3-4A proteinase: NS3 is a multifunctional protein that contains a serine proteinase domain in its 180 N-terminal amino acids. The remainder of the protein encompasses an RNA helicase. The NS3 proteinase belongs structurally to the trypsin superfamily but is unique in requiring a non-catalytic, structural zinc atom and a second viral protein as a cofactor. The proteinase cofactor, NS4A, is a relatively small protein, consisting of only 54 residues. The first 20 residues of NS4A are highly hydrophobic and are believed to be involved in membrane anchoring of the NS3-4A proteinase/helicase complex. The function of the hydrophilic 20 C-terminal residues of the cofactor is presently unknown, whereas the central residues of NS4A, amino acids 21–34, were shown to interact directly with NS3 and to be absolutely required for the enhancement of its serine

proteinase activity. The structural zinc ion is coordinated by residues located opposite to the active site. Bound zinc is believed to play an essential structural role, as its removal was shown to lead to unfolding and precipitation of the protein.

Interfering with zinc or NS4A binding would be a strategy to inhibit the NS3-dependent serine proteinase activity. Targeting these sites selectively with small, drug-like molecules is currently viewed as extremely difficult, however. Of the possible mechanisms of inhibition of the NS3 proteinase, the one that holds the most promise is inhibition of substrate binding. The NS3-dependent cleavage sites of the HCV polyprotein have the consensus sequence Asp/Glu-(Xaa) 4-Cys/Thr↓Ser/Ala-(Xaa)2-Leu/Trp/Tyr, with cleavage occurring after cysteine or threonine. Analysis of the cleavage kinetics of different peptide substrates has shown that the minimum length required for a synthetic substrate is a decamer incorporating all of these conserved features and spanning six amino acids upstream to four amino acids downstream of the P1-P1′ cleavage site. The nomenclature of Schechter and Berger in designating the cleavage sites as P6-P5-P4-P3-P2-P1….P1′-P2′-P3′-P4′-, with the scissile bond between P1 and P1′ and the C-terminus of the substrate on the prime side. The rather unusual requirement for large peptide substrates can be explained through the structural analysis of the substrate-binding site as revealed by the three-dimensional structure of the enzyme; the substrate-binding channel is strongly cationic, solvent-exposed, and relatively featureless. Selective recognition of the substrate is derived from a series of weak interactions that are distributed along an extended contact surface and that involve all of the evolutionarily conserved features of the cleavage site sequences. This architecture has

made the design of potent, small molecular weight inhibitors challenging. Despite this anticipated difficulty, compounds that block replication of HCV replicons in cell culture have been reported. The antiviral effect of an NS3 proteinase inhibitor, termed BILN-2061, has recently been shown in early, proof-of-concept clinical trials. As described below, a number of peptide-based or peptidomimetic inhibitors have been developed for the NS3-4A proteinase. Most of these inhibitors fall in one of three classes: (1) substrate analogues, (2) inhibitors containing a covalent serine trap, and (3) product-like inhibitors. In addition, a few non-peptidic small molecule inhibitors have been recently reported.

In substrate specificity studies identified P1′ substitutions with the amino acids proline, tetrahydroisoquinoline-3-carboxylic acid (Tic) or pipecolinic acid (Pip), respectively, that abolished cleavage but retained a high affinity of the corresponding peptides for the proteinase. The decapeptide Glu-Asp-Val-Val-Leu-Cys-Tic-Nle-Ser-Tyr was reported to be a potent, competitive inhibitor of the NS3-4A proteinase. The residues corresponding with the P′ portion of the noncleavable substrate analogues in order to optimize binding to the substrate binding cleft. Their effort led to a substrate-derived peptide inhibitor of the NS3-4A proteinase with the amino acid sequence Asp-(D) Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu. This peptide displayed a more than three orders of magnitude increase in potency relative to the starting peptide. Although a great deal of selectivity and potency can be obtained through the design of substrate analogues, their peptidic nature and relatively large molecular weight limits cell-membrane permeability and bioavailability, thus preventing employment in clinical trials.

Serine-trap inhibitors: Serine proteinase inhibitors can typically be developed by

derivation of the known substrate by replacing the scissile amide bond with an electrophilic "warhead" able to form a covalent adducts with the catalytic serine residues. Compounds of this mechanistic class are often referred to as "transition-state analogues" or "serine-trap inhibitors." Several pharmaceutical groups have reported a series of electrophile-based inhibitors, which have included alpha-keto amides, boronic acids, hydrazinoureas, and alpha-keto acids.

Product analogues: The NS3 proteinase is susceptible to feedback inhibition by the N-terminal products released from the polyprotein substrate after enzymatic cleavage. The hallmark of protease N-terminal products and product-based inhibitors is the presence of a free carboxylic acid on the C-terminal P1 residue. This carboxylic group is liberated by the cleavage of the peptide bond and is believed to establish crucial and unique interactions with the enzyme-active site. This hypothesis is supported by recent structural data for full-length NS3 protein crystallized as an enzyme-product complex. In this case, the C-terminal threonine of the NS3 helicase domain, which represents the N-terminal product of the NS3-N4A cleavage event, was found occupying the active site of the proteinase domain. The significance of this pronounced product feedback inhibition with respect to polyprotein processing or viral replication is presently unknown. Based on the observation of the product inhibition phenomenon, two groups have systematically modified the natural amino acids in these product inhibitors in order to obtain highly potent hexapeptide inhibitors of the NS3-4A proteinase. The C-terminal carboxylic acid plays a critical role in determining the affinity and selectivity of product-based inhibitors of the NS3-4A proteinase and was exploited as an active-site anchor to develop a new

generation of potent, selective tripeptide acid and peptidomimetic product-based inhibitors. Macrocyclic peptidomimetic compounds in this series represented by (compound 7) have been reported to inhibit the HCV-1b and HCV-1a proteases with inhibition constant values in the low nanomolar range. Submicromolar concentrations of these compounds were inhibitory in both a cell-based NS3-dependent reporter as well as replicon assays.

Non-peptide inhibitors: The limitations of peptides and peptide derivatives as drugs are known. Therefore, concurrent with reducing the size and peptide nature of the various inhibitors and efforts to discover non-peptidic inhibitors were also made. Selected examples of a rhodanine derivative bisbenzimidazole derivative. The detailed mechanism of these inhibitors and their potential to inhibit HCV replication in cell-culture or animal models remains to be established before considering these compounds as potential clinical candidates.

The viral replication complex: The exact composition of the viral replicase complex is not known. It is often assumed that all of the viral nonstructural proteins are present in a membrane-bound ribonucleoprotein complex termed the “replication complex.” Two enzymatic activities have been identified that are likely to be involved directly in genomic RNA replication: an NTPase/helicase activity residing in the C-terminal two thirds of NS3.

NS3 helicase inhibitors: The HCV NS3 protein contains a RNA helicase domain in the C-terminal 500 amino acids. This region of NS3 contains an Asp-Glu-Cys-His (DECH)

motif that identifies it as a member of the DEXH subfamily of DEAD (Asp-Glu-Ala-Asp) box helicases. The NS3 helicase is an enzyme capable of unwinding duplex RNA; the energy required for the unwinding reaction is believed to be generated by the hydrolysis of nucleoside triphosphates. Nucleic acid-stimulated NTPase activity has been shown to be an additional property of the NS3 helicase. The NS3 helicase activity is presumably involved in the resolution of double-stranded replicative intermediates generated during the replication of the HCV RNA genome.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is described as the ELISA reactivity of BVDV NS3/NS4a antibodies to HCV antigens.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Selection of Nucleic acid antigens was performed on the following HCV and BVDV isolates Hepatitis C virus subtype 1a : Hepatitis C virus 1a (isolate 1) , Hepatitis C virus 1a (isolate H), Hepatitis C virus 1a (isolate H77); Hepatitis C virus subtype 1b : Hepatitis C virus 1b (isolate BK), Hepatitis C virus 1b (isolate Con1) , Hepatitis C virus 1b (isolate HC-JT), Hepatitis C virus 1b (JFH-1) , Bovine viral diarrhea virus-1 (Cytopathic) : NADL , Singer , C24V , M3 (Minn.), 5960 (Iowa), 85-468 (S. Dak.) , 86-6998 (S. Dak.) , 86-7061 (S. Dak.) , 86-7383 (S. Dak.) , 87-2552 (Iowa) , 87-2558 (S.

Dak.), 88-9132 (Minn.), 12938 (Colo.) , 19043 (Colo.), 19394 (Colo.) , 21372 (Colo.) , Bovine viral diarrhea virus-1 (Noncytopathic) : NY-1 , Draper Neb. (Nebr.) , 85-13507 (Wis.) , 85-14374 (S. Dak.) , 86-225 (S. Dak.) , 86-888 (S. Dak.), 86-1473 (S. Dak.) , 86-1618 (S. Dak.), 86-4656 (S. Dak.) , 86-7860 (Iowa), 86-9545 (S. Dak.), 86-14462 (S. Dak.) , 86-98119 (Kans.) , 87-799 (Iowa) , 87-1381 (S. Dak.) , 87-1411 (Minn.), 87-1795 (S. Dak.), 87-1882 (S. Dak.) , 87-1888 (S. Dak.) , 87-2111 (S. Dak.) , 87-2127 (S. Dak.) , 87-3810 (S. Dak.) , 88-595 (S. Dak.).

BVDV NS3/NS4A

GPAVCKKITEHEKCHINILDKLTAFFGIMPRGTTPRAPVRFPTSLLK

VRRGLETAWAYTHQGGISSVDHVTAGKDLLVCDSMGRTRVVCQSNNRLTDETE

YGVKT

DSGCPDGARCYVLNPEAVNISGSKGAVVHLQKTGGEFTCVTASGTPAFFDLKNL

KGWS

GLPIFEASSGRVVGRVKVGKNEESKPTKIMSGIQTVSKNRADLTEMVKKITSMNR

GDF

KQITLATGAGKTTELPKAVIEEIGRHKRVLVLIPLRAAAESVYQYMRLKHPSISFN

LR

IGDMKEGDMATGITYASYGYFCQMPQPKLRAAMVEYSYIFLDEYHCATPEQLAI

IGKI

HRFSESIRVVAMTATPAGSVTTTGQKHPIEEFIAPEVMKGEDLGSQFLDIAGLKIP

VD

EMKGNMLVFVPTRNMAVEVAKKLKAKGYNSGYYYSGEDPANLRVVTSQSPYV
IVATNA

IESGVTLPDLDTVIDTGLKCEKRVRVSSKIPFIVTGLKRMAVTVGEQAQRRGRVG
RVK

PGRYYRSQETATGSKDYHYDLLQAQRYGIEDGINVTKSFREMNYDWSLYEEDSL
LITQ

LEILNNLLISEDLPAAVKNIMARTDHPEPIQLAYNSYEVQVPVLFPKIRNGEVTDT
YE

NYSFLNARKLGEDVPVYIYATEDEDLAVDLLGLDWPDPGNQQVVETGKALKQV
TGLSS

AENALLVALFGYVGYQALSKRHVPMITDIYTIEDQRLEDTTHLQYAPNAIKTDGT
ETELKELAS

HCV-1 was a positive-sense RNA virus with approximately 9,400 ribonucleotides, containing a poly(A) tail at the 3' end. The sequence contained a 5' untranslated region (5' UTR) of 341 bases, a long open reading frame coding for a polyprotein of 3,011 amino acids, and a 3' untranslated region (3' UTR) of about 27 bases. This RNA structure is most similar to that of the family Flaviviridae, which encompasses numerous arthropod-borne viruses. Consistent with the known functions of most Flavivirus proteins, the three N-terminal HCV proteins are probably structural (C, E1, and E2/NS2) and the four C-terminal proteins (NS2, NS3, NS4, and NS5) are believed to function in viral replication.

Hepatitis C virus (HCV) and bovine viral diarrhea virus (BVDV) are both members of the Flaviviridae family and share many molecular and virological similarities (Lindenbach, B. D., and C. M. Rice. 2003. Molecular biology of Flaviviruses. Adv. Virus Res. 59:23-61 and Rice, C. M. 1996. Flaviviridae: the viruses and their replication, p. 931-959. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology. Lippincott, Philadelphia, Pa. They both have single-strand RNA genomes that replicate via negative-strand intermediates and produce a single polyprotein that is cleaved into individual proteins by a combination of host and viral proteases. Although HCV is a Hepacivirus and BVDV is a Pestivirus, there is a low degree of sequence homology between their respective nonstructural proteins and both RNA-dependent RNA polymerases are structurally very similar Choi, K. H., J. M. Groarke, D. C. Young, R. J. Kuhn, J. L. Smith, D. C. Pevear, and M. G. Rossmann. 2004. The structure of the RNA-dependent RNA polymerase from bovine viral diarrhea virus establishes the role of GTP in de novo initiation. Proc. Natl. Acad. Sci. USA 101:4425-4430.

HCV is a major etiological agent for viral hepatitis. The World Health Organization estimates that 170 million people are chronically infected with HCV worldwide and of those, 4 million are in the United States. Within 10 to 20 years of infection, 20 to 30% of chronic carriers develop cirrhosis, making HCV infection one of the major reasons for liver transplantation. Current therapies are limited to interferon treatment, either alone or in combination with ribavirin (Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling, and J. K. Albrecht. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet

358:958-965. and Walker, M. P., T. C. Appleby, W. Zhong, J. Y. Lau, and Z. Hong. 2003. Hepatitis C virus therapies: current treatments, targets and future perspectives. Antivir. Chem. Chemother. 14:1), but patient response for certain genotypes is still unsatisfactory. This unmet medical need has created an urgent demand for the development of new drugs to treat chronic hepatitis C. However, the lack of an in vitro infection system has hampered HCV drug development. For this reason, BVDV has sometimes been used as a surrogate infectivity model in vitro.

BVDV infection represents an economically important disease of cattle. Infection of cattle with BVDV leads to a variety of disorders ranging in severity from subclinical or mild to fatal. The virus is classified into two biotypes, cytopathic (cpBVDV) and non-cytopathic (ncpBVDV), based on their effects in tissue culture. cpBVDV induces a type I interferon response in bovine macrophages (Adler, B., H. Adler, H. Pfister, T. W. Jungi, and E. Peterhans. 1997. Macrophages infected with cytopathic bovine viral diarrhea virus release a factor(s) capable of priming uninfected macrophages for activation-induced apoptosis. J. Virol. 71:3255-3258. and leads to apoptotic cell death in cultured cells and Hoff, H. S., and R. O. Donis. 1997. Induction of apoptosis and cleavage of poly(ADP-ribose) polymerase by cytopathic bovine viral diarrhea virus infection. Virus Res. 49:101-113. However, ncpBVDV-infected macrophages do not produce type I interferon, and infected cells do not respond to double-stranded RNA treatment Schweizer, M., and E. Peterhans. 2001. Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J. Virol. 75:4692-4698. Indeed, the activity of the double-stranded RNA analog, poly (I) · poly(C), against vesicular stomatitis virus is inhibited by ncpBVDV coinfection (Rossi, C. R., and G. K. Kiesel.

1983. Characteristics of the polyriboinosinic acid: polyribocytidylic acid assay for noncytopathogenic bovine viral diarrhea virus. Am. J. Vet. Res. 44:1916-1919. Infection of cells with ncpBVDV can enhance the replication of other viruses by blocking the production of interferon. Inaba, Y., Y. Tanaka, T. Kumagai, T. Omori, and H. Ito. 1968. Bovine diarrhea virus. II. END phenomenon: exaltation of Newcastle disease virus in bovine cells infected with bovine diarrhea virus. Jpn. J. Microbiol. 12:35-49. Production of NS3 generally correlates with cytopathogenicity (Meyers, G., and H. J. Thiel. 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47:53-118. In ncpBVD, cleavage at the NS2/3 site does not occur and only uncleaved NS2/3 is observed. In the case of cpBVDV biotypes, both NS3 and NS2/3 products are found in virus-infected cells.

Vector and BVDV gene for NS3/NS4a cDNA Insert: pGX2T vector, an expression system based on the cDNA, has been obtained from Pharmacia, NJ. Bovine Viral Diarrhea Virus-1 cDNA encodes for NS3/NS4a were cloned into pGx2T vector, at BamH1 site for expression as a fusion protein in JM109 E. coli. The plasmid vector contains the glutathione-S-transferase (GST) gene under the control of TAC promoter. A recombinant bacterium carrying gene for NS3/NS4a was multiplied in LB broth, and the bacterial pellet was collected by centrifugation at 4000 X g for 10 min. The polypeptides were isolated from the bacterial pellet, denatured by urea, and renatured with dialysis in PBS. The amount of protein was determined by a BioRad DC protein assay kit. The purified polypeptides were analyzed by SDS-PAGE followed by immunoblotting. The polypeptides transferred onto nitrocellulose paper were probed with a 1:100 dilution of

human HCV-positive sera and negative sera. Then affinity-purified peroxidase-labeled goat anti-bovine IgG (H+L) (KPL Inc. Galthersburg, MD) was added, and the nitrocellulose papers were developed for color reaction in 4-Chloro-1-naphthol with 30% H_2O_2.

Electroporation and Recombinant Plasmid DNA: The recombinant Pgx2t-NS3/NS4a cDNA was electroporated into competent bacterial cells (Max Efficiency *E. coli*-JN109; Promega, WI). The presence of NS3/NS4a was confirmed on the plasmid DNA by running agarose gels to determine the presence of high molecular weight recombinant DNA in comparison with the vector without insert. The extracted recombinant DNA from bacteria was tested for the presence of BVDV-NS3/NS4a gene for correct orientation on pGX2T vector. The various sites on NS3/NS4a gene were studied using SEQIAD computer gene sequence analysis developed at Kansas State University, Manhattan, KS and Collett et.al. 1988a, 1988b. Virology 165 191-199.

Preparation of pGX2T-NS3/NS4a DNA for intramuscular inoculation of mice: Qiagen anion exchange columns, supplies and procedures were used for purification of DNA (QIAGEN Inc, Santa Clarita, CA). Seven groups of 10 female blub/c mice (5-8-week-old) (70 mice total) were injected with recombinant protein NS3/NS4a DNA (100 and 200 μg) belongs to Group 1 and Group 2. Group 3 and 4 received vector-NS3/NS4a DNA (100 and 200 μg), group 5 and 6 received vector control plasmid (100-200 μg) or E.coli proteins without NS3/NS4a and group 7 were injected with PBS. Specifically, the quadriceps muscles of the mice were injected with 100 μl of 0.5% bupivacaine

hydrochloride and 0.1% methylparaben in isotonic sodium chloride. Twenty-four hours following the bupivacaine injection, 100-200 µg of the pGX2T-NS3/NS4a or pGX2T was injected into the same site at two weeks interval for a total of four inoculations. Recombinant NS3/NS4a protein was immunized intramuscularly (i.m.). Control mice were immunized with the parental pGX2T vector or E.coli proteins without NS3/NS4a. Six weeks after the first immunization, all the mice were humanly sacrificed, and the sera and spleen cells were pooled from each group for study of the immune responses generated against HCV.

Cell-mediated cytotoxicity (CTL): The assay has been described in detail previously Sallberg et al., J. Virol. 71:5295 (1997. To study whether the constructs described above were capable of eliciting a cell-mediated response against NS3/NS4a, an in vivo tumor growth assay was performed. To this end, an SP2/0 tumor cell line stably transfected with the NS3/4a gene was made. The Pgx2T plasmid containing the NS3/4a gene was linearized by BamH1 enzyme restriction digestion. The 5 µg linearized plasmid DNA was mixed with 60 µg transfection reagent and the mixture was added to a 50% confluent layer of cells of the myeloma cell line Sp2/0 in a 35 mm dish were cultured in RPMI media (Gibco), supplemented with 10%FCS (Sigma, USA) 100u/ml penicillin (Gibco), 100mkg/ml streptomycine (Gibco) (R10). The transfected SP2/0 cells (NS3/4a-SP2/0) were grown for 15 days and individual clones were isolated. A stable NS3/4a-expressing SP2/0 clone was identified using PCR and RT PCR. The cloned cell line was maintained in DMEM containing 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin.

The in vivo growth kinetics of the SP2/0 and the NS3/4a-SP2/0 cell lines were then evaluated in Balb/c mice. Mice were injected subcutaneously with $2x10^6$ tumor cells in the left flank. Each day the size of the tumor was determined through the skin. The growth kinetics of the two cell lines was comparable. The mean tumor sizes did not differ between the two cell lines at any time point. The experiments that were performed to determine whether mice immunized with the NS3/4A constructs had developed a T-cell response against NS3/NS4a.

Lymphoproliferative responses in immunized mice: To examine whether a T-cell response is elicited by the NS3/4a immunization, the capacity of an immunized mouse's immune defense system to attack the NS3-expressing tumor cell line was assayed. The protocol for testing for in vivo inhibition of tumor growth of the SP2/0 myeloma cell line in Balb/c mice has been described in detail previously by Encke et al., J. Immunol. 161:4917 (1998).

To determine whether NS3/NS4a-specific T-cells were elicited by the NS3/4a immunizations, an in vitro T-cell mediated tumor cell lysis assay was employed. The assay has been described in detail previously (Reddy et.al, Comp. Immu. Micro. Infec. Dis. 1999, 22(4): 231-246). Percent specific lysis was calculated as the ratio between lysis of NS3/4a- pGX2T SP2/0 cells and lysis of SP2/0 cells. Only mice immunized with NS3/4a- displayed specific lysis over 10% in four out of five tested mice, using an effector to target ratio of 20:1.

Assays of humoral immune response : Sera obtained from each group of mice were pooled and diluted (twofold) in PBS and analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) using plates coated with Hepatitis C antigens (Commercial kits purchased from Abbott Laboratories, Inc) and also tests on NS3/NS4a expressed r-protein in E.coli or E.coli proteins. Sera from immunized mice as well as were pooled and tested for antibody response to BVDV-NS3/NS4a by ELISA and Western Blots. The antibody responses in sera of immunized mice to NS3/NS4a were analyzed by the procedures similar to that already described (Xu et. al., 1997, J. Virolgy 1997, 71(7) 5312-22).

In addition, HCV-positive sera and negative sera obtained from n=87 patients either infected with HCV 1a (n=67) or 1b (n=20) were analyzed by ELISA and Western Blots.

Statistical analysis: Test for significant correlation between compared treatments was carried out using the Mantel-Haenszel correlation test. A significant level of a = 0.05 was used for all tests. The Goodman and Kruskalis g-statistics were used to measure the correlation between the treatments. All analyses were carried out using the SAS 'FREQ' procedure (SAS Institute, Inc. 1998).

Genetic inoculation induces humoral immunity in mice: Mice immunized with pGX2T-NS3/NS4a r-protein or DNA developed an increase of the antibody to HCV NS3/NS4a. In ELISA, the average optical density (OD) value for pGX2T -NS3/NS4a was 1.1 in contrast with the vector control (OD=0.3). Statistical analysis of HCV-NS3/NS4a r-protein or DNA specific antibody responses were significant ($p =< 0.001$) in mice

compared with vector control or E.coli proteins. The pre-immune sera from the non-inoculated animals or PBS inoculated, as well as from control mice inoculated with the identical plasmid backbone, failed to show reactivity. The sera from mice inoculated with pGX2T-NS3/NS4a construct reacted positively with recombinant r-protein and HCV ELISA commercial test kits and Western Blots contain r-protein of NS3/NS4a. The pre-immune sera from the non-inoculated animals, as well as from control mice inoculated with the pGX2T plasmid without NS3/NS4a insert failed to show reactivity. The immune responses of all animals were consistent. A single inoculation of 100 μg of a construct or r-protein, which expresses BVDV-NS3/NS4a, resulted in the seroconversion of 100% of the animals after inoculation.

Particularly evasive are the hepatitis viruses, which are not classified as a family but are grouped based on their ability to infect cells of the liver. Whereas Bovine Viral Diarrhea Virus of Cattle and Wild Animals, Hog Cholera virus of Swine, Border Disease of Sheep, Dengue Virus 2 and Yellow Fever Virus of human that include Hepatitis C Virus (HCV) belongs to the Flaviviridae family of single-stranded RNA viruses. (Virology, Fields ed., third edition, Lippencott-Raven publishers, pp 945-51 (1996). The genomic RNA of Flaviviruses is translated into one single polyprotein that is subsequently cleaved by viral and cellular proteases to yield the functional polypeptides. The polyprotein is cleaved to three structural proteins (core protein, E1 and E2), to p7 of unknown function, and to six non-structural (NS) proteins (NS2, NS3, NS4A/B, NS5A/B). NS3 encodes a serine protease that is responsible for some of the proteolytic events required for virus maturation and NS4A acts as a co-factor for the NS3 protease. NS3 further displays

NTPase activity, and possesses RNA helicase activity in vitro.

Pathogenicity of HCV typically progresses from an acute to a chronic phase. Acute infection is characterized by high viral replication and high viral load in liver tissue and peripheral blood. The acute infection is cleared by the patient's immune defense system in roughly 15% of the infected individuals; in the other 85% the virus establishes a chronic, persistent infection. (Lawrence, Adv. Intern. Med., 45:65-105 (2000). During the chronic phase replication takes place in the liver, and some virus can be detected in peripheral blood. (Virology, Fields ed., third edition, Lippencott-Raven publishers, 1042 (1996). The HCV pathogenicity when compare to Bovine viral diarrhea virus, a major viral pathogen of cattle Worldwide, causes disease characterized by immunosuppression and lymphocytopenia. In addition, infection of a fetus in the first 150 days of pregnancy can give rise to a persistently infected calf that sheds the virus for life. This is a major means of dissemination of the virus to naïve animals and herds. BVDV is lymphotrophic and acute infection results in depletion of lymphoid tissues, even with BVDV strains of low virulence.

Essential to the establishment of a persistent infection of HCV is the evolution of strategies for evading the host's immune defense system. HCV, as a single stranded RNA virus, displays a high mutation rate in the replication and transcription of its genome. Thus, it has been noted that the antibodies produced during the lytic phase seldom neutralize virus strains produced during chronic infection.

The infected host mounts both a humoral and a cellular immune response against the HCV virus but in most cases the response fails to prevent establishment of the chronic disease. Following the acute phase, the infected patient produces antiviral antibodies including neutralizing antibodies to the envelope proteins E1 and E2 due to antigenic variability among HCV genotypes and subtypes within a genotype. Several attempts were made during several years using HCV NS3/NS4 homologous or conserved genes and antigens to prevent HCV. Our studied showed that conserved genes among genotypes and subtypes of HCV also display high mutation in the replication and transcription of its genome.

Whereas in BVD virus such high mutation on conserved or non-conserved genes and antigens is limited or low compare to HCV. The very reason we used BVDV conserved antigens similar to HCV were used to prevent HCV infection in vivo and vitro animal experimental models.

CLAIMS

I Claim:

1. A purified or isolated nucleic acid consisting of the sequence of BVDV NS3/NS4a for preparation of Vaccine against all Hepatitis C genotypes and sub types for the prevention of human Hepatitis C infections.

2. A vector comprising the nucleic acid of claim 1.

3. An immunogenic composition comprising the nucleic acid of claim 1.

4. A purified or isolated nucleic acid of BVDV expressed as fusion or non-fusion protein or proteins in bacteria and other eukaryotic, prokaryotic, insects and mammalian cells for the use in prevention of Hepatitis C genotypes 1a and 1b in human.

5. An isolated r-protein comprising the nucleic acid of claim 4.

6. An immunogenic composition comprising the r-protein of claim 4.

7. A method of producing HCV vaccine using bovine viral diarrhea virus NS3/NS4a, the method comprising, the steps of;

1. screening the nucleic acid sequences of bovine viral diarrhea viruses and HCV-1 a and 1b cDNA encodes for NS3/NS4a ;
2. insertion of NS3/NS4a cDNA gene into expression vector;
3. isolation of expressed protein;
4. confirmation of the expressed gene using gene sequence analysis; and
5. observing humoral and cell mediated immune response using mouse models.

8. The methods of making and using the compositions of BVDV NS3/NS4a including DNA and r-protein, but not limited to, diagnostics and medicaments for the treatment and prevention of HCV infection caused by the HCV genotypes 1a and 1b as claimed in claim 7.

9. The method of eliciting humoral and cell mediated immune response against Hepatitis C virus in animals and human as claimed in claim 7.

10. The method of production of Hepatitis C vaccine using BVDV NS3/NS4a for human use, not limited to laboratory or at industrial scale production as claimed in claim 7.

11. The method of production of Hepatitis C vaccine derived from Bovine Viral Diarrhea Virus (BVDV) -1 and 2 that includes cytopathic and non-cytopathic isolates from cattle as claimed in claim 7.

ABSTRACT

The current invention draws scope of newer methodologies in the development of vaccine against Hepatitis C virus genotype-1a and b. On the basis of phylogenetic analysis of nucleotide sequences of NS3/NS4a genes of multiple genotypes and subtypes of Hepatitis C virus (HCV) and Bovine Viral Diarrhea Virus (BVDV) strains has been identified. Characterization of these genetic groups is facilitated and contributed to the development of an effective NS3/NS4a vaccine against HCV genotype-1 a and b. This invention is directed to NS3/NS4a protein derived from conserved regions of BVDV cloned into E.coli bacteria for the production recombinant BVDV NS3/NS4a protein. The immunization of r-NS3/NS4a protein or DNA of BVDV induced humoral and cell mediated protective immune response against Hepatitis C viruses 1a and 1b in mouse models.