Editorial Commentary

Antigen glycosylation broadens the immunogenicity of E2 subunit HCV vaccines

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Wang X, Yan Y, Gan T, *et al.* A trivalent HCV vaccine elicits broad and synergistic polyclonal antibody response in mice and rhesus monkey. Gut 2019;68:140-9.

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Introduction

Globally, an estimated 71 million people are chronically infected with hepatitis C virus (HCV), and 400,000 people died mostly from HCV-related cirrhosis and liver cancer. Recent implementation of direct-acting antivirals (DAAs) targeting HCV nonstructural proteins has greatly increased the cure rate overall up to 90% of chronic hepatitis C patients, thus some optimistically consider that HCV is amenable to eradication and less research interest remain for HCV. However, such considerations have neglected several important issues, which includes large number of HCV-related deaths annually, unawareness of infection, low access to diagnosis and treatment, limited sterilizing immunity, risk of reinfection and ceaseless progression to liver cancer after DAA treatment. Thus, the burden of hepatitis C will continue to be a major public health problem. An effective prophylactic HCV vaccine is clearly and undisputedly required to achieve the global elimination HCV infection, a part of global hepatitis goals under the Sustainable Development Agenda 2030 set by the World Health Organization (WHO) in 2016.

Vaccine candidates tested with disappointing results

To date, there is no licensed vaccine for HCV. Since discovery of HCV in 1989 (1), tremendous efforts have been invested to test a number of prophylactic vaccine candidates against HCV infection. Diverse strategies have been applied to induce humoral and cellular responses, including DNA vaccines, virus-like particles (VLPs), recombinant proteins, adenovirus (Ad) vectors, and inactivated whole HCV particles in rodents, nonhuman primates, or human volunteers (2-4). However, only a few have been tested in clinical trials and unfortunately none are successful.

Full-length E2 or E1E2 recombinant proteins could induce heterologous neutralizing activity in guinea pigs (5), and protected chimpanzees against homologous HCV challenge (6), but only elicited detectable broad neutralizing antibodies (bNAbs) in a small portion of humans in phase 1a trials (7). Truncated E2 with deletion of either hypervariable regions (HVRs) or HVR1 induced moderate titers of bNAbs in guinea pigs (8). VLPs carrying core, E1, and E2 proteins of one or multiple genotypes hardly induce antibody

response (3,9). Inactivated whole HCV particles (J6/JFH-1) provided limit protection in mice with humanized hepatocytes (10). Nonstructural proteins (NS3, NS4, NS5), with or without inclusion of envelope proteins, were designed to induce cellular immune responses. Such vaccines composed of a replication-defective chimpanzee adenovirus (ChAd) vector have been tested in phase I trials in human volunteers (11). ChAd3 expressing the nonstructural proteins (ChAd3-NS) was found to induce long-lasting T- and B-cell memory responses in mice and macaques, but in phase I trials it induced a higher CD8 T cell response but a lower frequency of CD4 T cells (12). The ChAd3-NS vaccine with modified vaccinia Ankara (MVA)-NS boosting was moved into phase 1/2 study, however the results were disappointed (2,11). Besides, DNA vaccines designed to express envelope proteins or other viral proteins have been tested in various immunization procedures, including DNA-based immunization, DNA priming and protein boosting, or recombinant adenovirus priming and DNA boosting in rodents and chimpanzees (2). All these vaccine candidates induced disappointing humoral and cellular responses.

New march from fundamental steps: antigen selection, modification, and formulations

Accumulating evidences and lessons urge a rethink that more fundamental work may be required to optimize vaccine antigens towards an increased antigenicity and immunogenicity. Previous results have raised the concerns that the use of recombinant HCV proteins as vaccine may face incorrect folding and/or undesired modifications of the proteins (13). Thus, sequence selection, codon optimization, and protein expression system may be determinants for an antigen to potentially be a successful HCV vaccine.

Recently, four studies from Huang's and Zhong's groups in Institut Pasteur of Shanghai, China, reported that a soluble form of E2 (sE2) produced in insect cells possessed different glycosylation patterns and induced humoral and cellular responses in mice and macaques; high titers of bNAbs were elicited with neutralizing activity against HCV genotypes 1-6 recombinant viruses (14-17). Initially, Li *et al.* selected E2 sequence from genotype 1b strain Con1 and codon-optimized the truncated form of E2 [amino acids (aa) 384 to 661, sE2] (15). The sE2 was cloned into the baculoviral vector pMT/BiP/V5-HisA and transfected into Drosophila S2 cells. After blasticidin screening, a stable cell clone expressing sE2, sE2B3, was chosen for large-scale production. The sE2 was efficiently secreted and stable in culture supernatant (100 mg/liter; at least 9 days). The sE2 was readily purified to near homogeneity (45 *vs.* predicted 34 kDa) and was identified to be fully deglycosylated by PNGase F and also contain endo H-resistant glycan types. Such unique glycosylation of insect cell-derived sE2 was different from mammalian-derived sE2 and proven to be critical for its superior ability to induce bNAbs. Besides, the sE2 was properly folded into a conformation that could be efficiently bound by bNAbs, AR3A (targeting conformational epitopes) and AP33 (linear epitopes), and entry receptors, CD81 and SRB1. The unique glycosylation patterns of insect sE2 facilitated a better receptor recognition, thus enabling it to clock cell culture-derived HCV (HCVcc) infection *in vitro*.

Insect sE2 was found to exist in three forms of monomers, dimers, and megamers, different from mammalian cells-expressed sE2 primarily in monomeric and dimeric forms. BALB/c mice immunized with insect sE2 induced sE2-specific antibodies peaked at week 6 and remained a relative high level (above 1,000, week 22), and adjuvant FA and a homologous boost increased the titer. The oligomeric state of insect sE2, as well as deletion of HVR1, did not affect bNAb induction. Anti-sE2 sera (1/40 dilution) contained both AP33-like and AR3A-like bNAbs and were able to efficiently neutralize (neutralization 50%) almost all 12 HCVcc recombinants containing envelope proteins of genotypes 1–6 in Huh7.5.1 cells. HCVcc reconstitutes the most properties of those in authentic virions and has been proven suitable for antiviral drug and neutralization testing, a feasible platform producing results with great clinical relevance (18-23). sE2 elicited antigen-specific IFN-γ and IL-4 T cell memory. Furthermore, protection experiments were performed and found that the sE2 vaccine of sE2/alum formulation generated high-titer sE2-specific antibodies and largely protected humanized Rosa26-Fluc mice (n=6) from BiCre-Jc1 (genotype 2a) challenge. Taken together, this study started from a fundamental step in obtaining a codon-optimized antigen and expression of insect sE2 with unique glycosylation pattern and gained promising results as an HCV vaccine candidate for further investigation.

Given the success in inducing high-titer bNAbs with prophylactic effect in mice, Li *et al.* proceeded to evaluate the protective efficacy in non-human primates (14). They immunized rhesus macaques (10 males and 10 females; 3–6 years old) with sE2 using different adjuvants at months 0, 1, and 2, and boosted at month 5. Blood was examined

at months 6, 7, and 8. Both humoral response and T-cell responses were induced in rhesus macaques, and a shift from a T-helper type 1 (Th1) response to a Th2 response and long-lasting T-cell memory responses were observed. Interestingly, neutralization breadth correlated with sE2-specific IgG titer. The sE2 with adjuvant alum/CpG and alum/MPL possessed more AP33-like and AR3A-like bNAbs, and such bNAbs (month 6; 1/20 dilution) were able to neutralize the infection of HCVcc recombinants of genotypes 1–7. Since alum/MPL adjuvants have been approved for human use, combination of sE2/alum/MPL has the advantage to be advanced into clinical trials.

Although a sE2 from 1b elicited antibodies and T-cell responses against various HCV genotypes, it is still a major hurdle for an HCV vaccine to induce immunity against highly divergent HCV genotype isolates. To cope with this issue, Wang *et al.* expanded the studies to cover more diverse HCV antigens by multivalent sE2 including genotypes 1a (stain H77), 1b (Con1) and 3a (S52) (16). All three insect-derived sE2 preserved the activity to bind E2 receptor CD81 or antibodies AR3A and AP33, as observed in the early report by Li *et al.* (15). Female BALB/c mice (n=6) was intraperitoneally injected with monovalent or trivalent sE2 (weeks 0, 2, 4 and 8). Trivalent sE2-induced high E2-specific antibody titers and persisted for 44 weeks, reaching almost the lifetime of a mouse. Monovalent sE2 may induced unique sets of antibodies, which had a stronger binding activity to homologous than heterologous antigens but could synergistically inhibit HCV infection. However, the trivalent sE2-immunised sera displayed strong binding to each of three sE2 antigens, inferring a better antigenic breadth than the monovalent sE2. Mice sera (week 10; 1/40 dilution) had the strongest neutralizing activities against HCVcc recombinants of genotypes 1–7 and decreasing dosage of each sE2 component in the trivalent formulation did not reduce neutralization ability. Further, no apparent interference among individual components of trivalent vaccine was observed. The trivalent sE2-induced antibodies may function cooperatively in neutralization, particularly in the neutralization of genotypes 1b, 2a, and 3a viruses. Nevertheless, more studies are needed to uncover mechanisms of synergistic neutralizing effect.

They also investigated the trivalent sE2 in rhesus macaques (3 male and 2 females per group; 3–5 years old) by immunization at months 0, 1, 2, 3, 4 and 7 (16). Overall, the trivalent and monovalent sE2 elicited comparable levels of E2-specific antibody titers. Anti-trivalent sE2 IgG purified from sera (month 8) neutralized 14 HCVcc of genotypes 1–7, no obvious genotype-specific neutralization effect, and the IC_{50} values of the trivalent group was 2–5 folds lower than that of monovalent group (≤500 μg/mL) for most of isolates. Anti-trivalent IgG was more efficient than the anti-monovalent IgG for neutralizing 1a (H77), 2a (JFH1 and PR63cc), 3a (S52), 5a (SA13), and 6a (HK6a). The coefficients of variation of IC_{50} measurements showed that the trivalent sE2 vaccine elicited stronger and broader as well as more equipotent and uniform NAb response in rhesus macaques with varying genetic backgrounds. A reduced variability in neutralization is crucial for any vaccine using in humans. This may be achieved by that multivalent vaccine has a better ability to buffer the variations of assays and increasing coverage of T cell epitopes which will increase the chance of MHC presentation.

T cell response was detected in splenocytes and CD4 and CD8 T cells (month 8) of rhesus macaques, however some variations existed (16). In general, splenocyte IFN-γ response was stronger than IL-4 response. Both monovalent and trivalent vaccines elicited TNF-α and IL-2 responses in Con1-sE2 or cocktail sE2-stimulated CD4 T cells, but IFN-γ response was only seen in cocktail sE2-stimulated CD4 T cells of trivalent vaccine group. In contrast, CD8 T cell response was much weaker in the both monovalent and trivalent vaccine groups. In addition, although these sE2 vaccines are capable of inducing broad and synergistic-acting polyclonal neutralizing antibodies in mice and rhesus macaques, the neutralizing titers may be still low compared with those in chronically infected patients. Future improvement of multivalent antigens in the balance of humoral and cellular responses as well as in the increased titers may be explored.

Besides selection of proper antigens, improvement of immunogenicity of sE2 and NAb titers were also explored. Yan *et al.* designed and produced a fusion protein of Con1 insect sE2 and the *Helicobacter pylori* ferritin (amino acids 5–167) (sE2-ferritin) which could self-assembled into a nanoparticle (10 nm) (17). sE2 was displayed on the surface of sE2-ferritin nanoparticles. Importantly, sE2-ferritin not only had nearly natural conformation but also had better affinities than the sE2 alone to neutralizing antibodies AR3A and AP33 and receptor CD81. The binding activity to the sera of 36 (genotype 1b) and 18 (2a) patients was significantly better than sE2 alone. sE2-ferritin nanoparticle induced anti-HCV bNAbs more potently than sE2, as the ability of inducing antibody a medium dose (0.217 nmol) of sE2-ferritin were comparable to high dose (0.65 nmol) of sE2. Both sE2-ferritin and sE2 induced E2-specific antibody

titers >10^5, which persisted for ≥30 weeks, and sE2-ferritin group (week 14, 1/40 dilution) showed significantly higher neutralization activities against HCVcc recombinants of genotypes 1–6, with ID_{50} values 2–5 times higher than sE2 group. Decreased neutralization titer of the sera from week 30 was less pronounced. Thus, a novel approach of antigen displaying further increases the immunogenicity of insect-derived sE2.

Perspectives

These four studies systematically demonstrate that the selection of appropriate E2 antigens, production in insect cells with unique glycosylation patterns, formulation in multivalent, and nanoparticle displaying determine the titer and breath of neutralization antibodies. Unique glycosylation patterns of insect sE2 show great advantage in eliciting high titer of nNAbs in mice and nonhuman primates and protects the humorized mice from challenge. The coverage and immunogenetic activity could be increased by multivalent components and nanoparticles displaying, and importantly these approaches could be combined and further optimized in future study to develop, such as, nanoparticle-based multivalent HCV vaccines. To date, these efforts and positive results should be emphasized and encouraged, especially in the present time when clinical trials of various vaccine candidates have encountered a failure. Although it is still a need to further optimize the antigen coverage, immunogenicity, and probably formulation, the results of these four studies have reached the line where the protection efficacy should be immediately evaluated in a robust *in vivo* infection model. Before such ideal models are available, existing animal models, for example, chimpanzees (24), humanized mice (25), as well as common marmosets (26) could be the alternatives. The immunocompetent common marmosets were shown susceptible to HCV-CE1E2p7/GBV-B chimera virus and able to generate specific humoral and cellular immune responses (26), thus potentiating it a model for testing the glycosylated E2 vaccines. Taken together, these studies urge the development of an immunocompetent animal model that enable to robustly repopulate the complete HCV life cycle for vaccine development and the study of HCV pathogenesis.

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Footnote

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