Discussion
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This study presents an attempt to develop a genotype 3 based vaccine against Hepatitis C, with special reference to India. Vaccine development for hepatitis C has been a challenging task because of the extreme variability of sequences among different genotypes and the global genotype distribution pattern (Simmonds et al 2005). It was shown that HCV infection in chimpanzees does not elicit protective immunity against reinfection with even homologous strain of the virus (Farci et al 1992) raising utility of a vaccine. However, several attempts were subsequently made to develop a vaccine (Torresi et al 2011). So far, majority of the recombinant candidate vaccines have been predominantly generated from genotype 1 virus (Ray et al 2009, Folgori et al 2006). It is heartening to note that many of these vaccine attempts including those reaching clinical trials produced cross-genotypic immune responses (Punterio et al 1998, Torresi et al 2005, Ray et al 2009). Still, the fact remains that the vaccine-genotype specific antibodies are mainly produced.

The first question we asked was the choice of HCV genotype. In India, an estimated 12 to 13 million people are infected with HCV (Narhari et al 2009) with a predominance of genotype 3 (>70%) (Lole et al 2003, our unpublished data). Therefore, the obvious choice was genotype 3. To address the issue of the geographic variation in genotype 3 sequences in India with well-known diversity, we sequenced samples from all over the country and considered these sequences to obtain the consensus based recombinant candidate vaccine.

The two components of our candidate vaccine were the hyper variable region 1 (HVR1) and non-structural protein-3 (NS3).

An effective vaccine will be the one that elicits substantial neutralizing antibody response to prevent viral entry into hepatocytes. Therefore, researchers have concentrated on virus proteins that are expressed on the surface of the virion and also involved in cell entry (Barth et al 2006, Callens et al 2005, Owsianka et al 2006, Wunschmann et al 2000). These proteins include the envelope glycoproteins E1 and E2.
The E2 glycoprotein harbours HVR1, a highly diverse region of the HCV genome. Taking into consideration the high mutation rate of the HVR1 region due to host as well as viral components (Caudai et al 2003, Chambers et al 2005, Shuhart et al 2006), we determined consensus HVR1 sequence based on the genotype-3 HCV sequences from different parts of India. For this, we used samples from all available subtypes of genotype 3, i.e. subtypes 3a, 3b, 3e, 3f, 3g and 3i, in the frequency at which they were detected in the population, 3a (27, 55.1%), 3b (8, 16.3%), 3e (2, 4.1%), 3f (2, 4.1%), 3g (8, 16.3%), and 3i (2, 4.1%).

The next issue to be addressed was the presence and distribution of HCV quasispecies in the samples studied. A shift over time of the minor quasispecies to the most predominant one has been reported earlier. Understanding the degree of variation and proportion of the different sequences was indeed an important exercise for us. For this, the quasispecies pattern was determined for all the 49 patients by sequencing 20 HVR1 clones obtained from every patient. The pattern varied from 2 to 14 quasispecies demonstrating degree of sequence variability in different patients and need to consider these variations for designing appropriate HVR1 peptides.

Amino acid positions 2, 6, 7, 10, 19, 20, 23, and 26 were found to be more conserved than the rest (91.8% at position 2 to 73% at position 10% conservation). Amino acid positions 11, 12, 14, 22 and 25 were found to be most variable (77.6% at position 14 to 63.3% at position 11). Importantly, except for position 10, all the other relatively more conserved amino acids were also present in the earlier consensus sequences reported earlier. The preference of amino acids in a given position of HVR1 provides information as to the combinations of permissible mutations in any position of HVR1 (Xiu et al 2003, Punteriero et al 1998). Upon comparing the consensus sequence of HVR1 obtained during this study with earlier available sequences, (Xiu et al 2003, Punteriero et al 1998), it was noted that the amino acids at positions 2(T), 5(T), 6(G), 7(G), 9(A), 12(T), 14(S), 15(G), 17(T), 18(S), 19(L), 20(F), 22(P), 23(G), and 26(Q) were conserved i.e. 15/27 (55.6%) positions were conserved. This significant observation encouraged us to proceed further and design possible
variants of the HVR1 sequence representing genotype 3 prevalent in the Indian population and also expect cross-reactivity with the other circulating genotypes. A total of 4 variants were thought to cover the range of sequence variability. Before assessing the immunogenecity of the 4 peptide variants, we examined immuno-reactivity with antibodies from 115 patients circulating different genotypes (genotypes 3, 1, 4 and 6), in ELISA. ELISAs were performed using the peptides individually, as a pool or as a single tetramer where the individual peptide sequences were used in tandem interspersed with 4 glycine residues.

Firstly, we used 105 anti-HCV negative samples to determine cut off value for anti-HCV positivity. With Mean O D value of NC + 3SD as the cut off value, false positivity varied from 0% to 2.9%. Overall, all the variants efficiently detected genotype 3 and a substantial proportion of genotype 1. No comments can be made with genotypes 4 and 6 with small numbers. Variants 3 and 4 exhibited higher reactivity than 1 and 2, variant 4 being the highest with respect to all the 4 genotypes (table 22). This variant differed from the consensus by a single amino acid at position 9, while 3 amino acids were different in variant 3 (positions 3, 8 and 9). Use of variant pool did not improve ELISA performance, in fact, resulted in lower reactivity than the individual variants 1, 3 and 4 probably because of the competitive inhibition. The tetramer exhibited lowest reactivity (42.6%) with the anti-HCV positive human serum samples when compared with the peptide pool (72.2%).

ELISA results showed that the HVR1 peptides used did exhibit a high degree of cross-genotypic reactivity and could be taken forward for the immunogenicity studies. Thus, we were ready with the first immunogen for the evaluation as candidate vaccine.

Let us now consider the second immunogen i.e., NS3 protein/gene. Clinical observations of HCV-specific immune responses in patients with acute self-limited HCV infection or patients who have recovered from chronic HCV infection suggest that T-cell mediated immune responses may determine the outcome of HCV infection (Rehermann 2000). An NS3 specific CD4+ T cell immune response is much stronger and more frequently found in patients who
resolve acute hepatitis than in patients who develop chronic infection and this response may be necessary for virus clearance (Diepolder et al 1995). Importantly, several studies have demonstrated the efficacy of the NS3 region as a vaccine candidate either singly or in combination with other HCV proteins (Jiao et al 2004, Fytili et al 2008, Pancholi et al 2003, Vajdy et al 2006, Zeng et al 2009).

In our study, we determined the immunogenicity of NS3 region along with HVR1. NS3 being a relatively conserved region, we decided to obtain the recombinant protein by amplifying and cloning the NS3 sequence from the serum of a chronically infected patient circulating HCV genotype 3a, the most predominant subtype-3 in India (Lole et al 2003, Narhari et al).

Unlike HVR1, the NS3 protein is relatively conserved across various subtypes and genotypes (Field's Virology, Simmonds et al 2005). We could show reactivity of the expressed protein with two heterologous anti-HCV positive sera, one belonging to genotype 1b, and the other to genotype 3a by western blot, thereby reaffirming the conserved nature of NS3.

As far as the first component of the candidate vaccine is concerned, it was amply evident based on different immunologic parameters that the use of synthetically developed HVR1 peptides generated promising results in mice. Various studies had demonstrated the immunogenic efficacy of peptides as vaccine candidates for HCV. Torresi et al (2006) used various combinations of the N-terminal 11 amino acids of HVR1 region to synthetically generate a lipidated peptide polytope and used it further for mice immunogenicity studies. Four long peptides from nonstructural proteins NS3, NS4 and NS5B containing HLA-class I and class II epitopes mainly inducing responses in natural infection were used to immunize HLA-A2.1 transgenic mice (Fournillier et al 2006). Interferon-gamma producing specific CD4 and CD8 cells were isolated from mice. In another study, two GB virus B (GBV-B) chimeric genomes, GBV-HVR1 and GBV-HVRh (with a hinge), containing the coding region of the immunodominant hypervariable region 1 (HVR1) of the E2 envelope protein were constructed and the RNA transcripts were used to inoculate two naïve
marmosets (Haqshenas et al 2007). These animals were able to clear HCV infection post challenge with HCV infected serum. In another study, 32 natural variants and 12 mimotopes of HVR1 region were used to generate a multiepitope peptide and further used to transfect B cell lines generated from HCV infected and healthy volunteers (Frasca et al 2003). Some HVR1 mimotopes were able to stimulate a multispecific CD4 T-cell repertoire that effectively cross-reacted with HVR1 native sequences. The study supported the use of HVR1 mimotopes in vaccines to prevent replication escape mutants.

Amongst the four variants, it was noted that HVR1 variant 2 was not immunogenic in mice. This variant differed from the consensus sequence by 5 amino acids (positions 3, 8, 9, 10, 11), while the variants 3 and 4 that differed from the consensus by 3 (positions 3, 8, 9) and 1 (position 9) amino acids respectively were immunogenic. Probably, substitutions at four consecutive positions or substitutions at positions 10 and 11 rendered variant-2 non-immunogenic. Since HVR1 variant 2 was antigenic against anti-HCV positive human sera, it was included along with other variants while generating the tetramer and variant pool.

In groups where mice were immunized with HVR1 variant pool, or HVR1 tetramer, antibody response against individual variants of HVR1 was assessed by coating each variant individually in ELISA. Anti-HVR1 antibody titres against individual variants 1, 2, 3 and 4 were comparable to the HVR1 variant pool and could be detected by all the variants individually, suggesting generation of cross-reacting antibodies.

On account of the inherent problems of the immunogenicity of peptides and recombinant proteins, use of adjuvants becomes necessary. In view of the crucial role played by various adjuvants in determining immunogenicity, (Choo et al 1989, Esumi et al 2002, Puig et al 2006), we decided to assess two adjuvants, liposomes and Al(OH)$_3$. Alum, the time-tested adjuvant was included as the "gold standard" because of the universal acceptance over a long time. In our lab, liposome was successfully used in the development of recombinant hepatitis E vaccine as judged by experiments in mice and rhesus monkeys
(Shrivastava et al 2010, Arankalle et al 2009) as well as for a recombinant combination vaccine for hepatitis E and B (Shrivastava et al 2010).

It has been shown earlier that liposome encapsulation enhanced immunogenicity of the HCV antigens (Jiao et al 2004, Engler et al 2004, Jiao et al 2003). With the encouraging results obtained by us earlier, liposome was an adjuvant of choice for hepatitis C. When we compared immune responses against the 27-amino acids HVR1 region with alum and liposomes (table-, figure-), alum always led to very low or no antibodies negating its utility. On the other hand, liposomes did prove extremely potent in inducing immune response against the peptides. No seroconversion was seen when HVR1 variant pool was given alone or with aluminium hydroxide as an adjuvant. However, 100% seroconversion was seen when HVR1 variant pool was given along with cadB adjuvant (table24). Titres of pHVR1 pool + pNS3 given along with cadB as adjuvant (mean log titre: 2.56±0.01) were significantly higher than those of pHVR1 pool + pNS3 given without adjuvant (mean log titre: 1.68±0.19) (p=0.002) (figure39) The antibody titres were significantly higher when tetramer was administered with cadB (mean log titre 2.28±0.11), than with aluminium hydroxide (mean log titre 0.94±0.11) (figure40). Though the tetramer induced high antibody titres with different formulations, the lower efficiency (42.6%, table22) in detecting anti-HCV antibodies in human serum samples argues against its suitability as a vaccine candidate.

Let us now consider the effect of adjuvants on the immunogenecity of recombinant NS3 protein of 70kda size. Being a protein of high molecular wt, it was weakly immunogenic even in the absence of any adjuvant (figure41, 42).

Formulations with 1µg pNS3, either alone (mean log titre 1.0±0.01) or given with cadB (mean log titre 1.65±0.11) did not show significant difference in antibody titres (p>0.05). Addition of DNA to 1µg pNS3 encapsulated in cadB (mean log titre 1.15±0.12) also did not result in significant increase in antibody titres (p>0.05). The anti-NS3 titres were significantly higher for pNS3-5µg-cadB group (mean log titres after 1st dose: 1.5±0.09, 2nd dose: 1.86±0.07, 3rd dose: 2.64±0.04) than pNS3-1µg-cadB group (mean log titres after 1st dose: 0.65±0.07,
2nd dose: 0.86±0.07, 3rd dose: 1.65±0.11) when compared for first (p=0.012), second (p=0.002) and third doses (p=0.009).

Adjuvanted pNS3 did produce significantly higher antibody titres. The effect with reference to adjuvant could be detected in NS3-HVR1 variant tetramer. 100% seroconversion was noted after third dose for tetramer-pNS3 given either with cadB or aluminium hydroxide, whereas 66.6% seroconversion was observed when NS3 was given with HVR1 variant pool or tetramer without any adjuvant.

As our aim was to use HVR1 and NS3 for reasons described earlier, it was important to assess the effect of adjuvants, the individual components and combination of these immunogens on the antibody response. Addition of pNS3 to the peptides did not alter anti-HVR1 antibody titres significantly (p>0.05) as compared to the addition of corresponding DNA (figures38, 39). Anti-HVR1-tetramer antibody titres were comparable for Tetramer-cadB (mean log titre: 2.28±0.11), Tetramer-pNS3-cadB (mean log titre: 2.53±0.05), and Tetramer-pNS3-aluminium hydroxide (mean log titre: 2.37±0.17) groups (p>0.05 for each comparison) (figure40). Thus addition of pNS3 did not affect anti-HVR1 antibody titres. As far as the antibody titres against NS3 component is concerned, addition of individual variants, variant pool or tetramer in Cad-B yielded optimum titres (figures41, 42).

As against our earlier observations of enhanced immune response following encapsulation of proteins and corresponding DNAs of hepatitis E and B viruses (Shrivastava et al 2009), no advantage was noted with both HVR1 and NS3. Thus, the phenomenon of enhancement of immunogenicity by DNA seems to be immunogen-dependent. Overall, for the generation of robust antibody titres against both the components of the candidate vaccine, Cad-B appeared to be the excellent choice.

Many reports of vaccine development for hepatitis C had used ELISPOT, LPA, intracellular staining and chromium release assays to determine the cell mediated responses against candidate vaccines (Fytilli et al 2007, Torresi et al...
In our experiments, we used cytokine bead array to determine the Th1/Th2 response pattern. We also substantiated this data by serum antibody isotype pattern, an indicator of the type of immune response obtained.

Earlier studies reported detection of IFN-gamma (Th1 response) and IL4 (Th2 response) using ELISPOT (Hallera et al 2007, Wüesta 2004). However, we did not observe strong T-cell mediated immune response as judged by the induction of cytokines by antigen-stimulated spleenocytes. In the cytokine bead array analysis, activation of both Th1 and Th2 cytokines was seen though at very low concentrations. This was probably because mice spleens were harvested at a later stage (15 days after 3rd dose). The possibility of higher elevation in these cytokines at early time points cannot be ruled out. Incidentally TNF-alpha the pro-inflammatory cytokine was detected at higher concentrations (to a maximum of 583.68±51.34 pg/ml).

Though cytokine data was inconclusive, the isotyping analysis did document involvement of both Th1 and Th2 type immune responses with NS3 and Th2 response with HVR1 components respectively (figures 44, 45, 46, 47). Variant-1 and 4 with 1 amino acid substitution behaved similarly i.e., Th2 response with 25µg variant+ corresponding DNA switching to balanced Th1/Th2 isotype pattern after the increase of peptide to 50µg or addition of 1µg pNS3. For HVR1 variant3, 50µg peptide + DNA group showed an increase in IgG2a titre than IgG1 titre (p=0.017) indicating Th1 bias. This difference in comparison to other HVR1 variants might have been because of the difference of three amino acids from the consensus sequence. Titres for other groups of HVR1 variant3 were comparable (p > 0.05) indicating a balanced Th1/Th2 type response.

In both HVR1 variant pool-DNA-cadB group (p=0.007) and HVR1 variant pool-pNS3-cadB group (p=0.010), IgG1 titres were significantly higher than IgG2a titres suggestive of Th2 bias. The immune response skewed towards Th2 type after the addition of either DNA or pNS3. tetramer-pNS3 cadB (p = 0.021) also. In this respect the HVR1 variant pool and tetramer behaved in a similar manner.
Overall, a balanced Th1/Th2 response was documented against pNS3. The addition of different HVR1 variants individually, or as pool or as tetramer did not alter the anti-NS3 Th1/Th2 balanced isotyping profile. Also, addition of DNA did not alter this response. Earlier results had documented a strong Th1 response against NS3 (Jiao et al 2004). However, we could detect both Th1 and Th2 responses. This would have been either because of the effect of adjuvants used or because of the low dose of pNS3 administered to the mice (1µg).

The effect of Al(OH)$_3$ and Cad-B adjuvants on isotype pattern could be studied only for variant-pool and tetramer. Individual peptides were not used to study the effect of adjuvants as our aim was to develop a candidate vaccine that would recognize maximum heterologous viruses circulating in the population. Hence, a combination of different variants would be essential. Al(OH)$_3$-variant-pool group remained antibody negative while addition of CadB led to balanced Th1/Th2 response (figure44). With tetramer, Al(OH)$_3$ gave balanced response while Th2 response was observed with CadB adjuvant (figure45). Thus the type of response was influenced by the adjuvant and immunogen used. Liposome encapsulation led to a skewing of immune response towards Th2 type irrespective of peptide pool or tetramer.

After adjuvanting peptides with Al(OH)$_3$ the mice remained antibody negative. With tetramer, a larger peptide, Al(OH)$_3$ could produce a balanced Th1/Th2 profile. Thus the enhancement of Al(OH)$_3$ seems to be peptide size dependent.

Earlier results for vaccine studies using HVR1 and NS3 as the immunogens documented the following results. HVR1 region was used either as an expressed protein, or as tandem repeats of HVR1 variants (Toressi et al 2007, Toressi et al 2009, Roccasecca et al 2005, Wei et al 2008). The immunogenicity of HVR1 was checked with anti-HVR1 antibody titres which were found to be comparable with those obtained during our study (Torresi et al 2007). Since HVR1 contains the neutralizing epitope, virus capture by anti-HVR1 antibodies was predominantly shown in various studies. (Torresi et al 2007, 2006). Less emphasis was laid on the Th1/Th2 immune response pattern. In our
study we could show a Th2 type immune response against HVR1. We also showed the binding of HCV belonging to genotypes 3a and 1b to the anti-HVR1 antibodies obtained from mice.

As far as HCV NS3 was concerned, studies have used both DNA and recombinant protein expressed either in bacterial or yeast system. Strong Th1 type of anti-NS3 response was detected in mice studies. (Jiao et al 2003, Jin et al 2007, Fytilli et al 2008). We could detect both Th1 and Th2 responses in mice thereby displaying a balanced response profile. This could have been because of the combination of HVR1 along with NS3 in our candidate vaccine. HVR1 being a part of structural protein E2 with strong Th2 response inducing ability, a balanced immune response pattern for the combination could have been obtained. The HVR1 peptides may have acted as an adjuvant to the NS3 region and vice-versa.

In the absence of an easy, efficient and convenient in-vitro / in-vivo neutralization test system, we performed the immune capture RT-PCR for the demonstration of binding of the virus to the antibodies generated against the HVR1. It is indeed significant to note that these antibodies were able to bind viruses of genotypes 3a and 1b. Whether this binding is indicative of neutralization remains to be established. Similar experiment was performed with HVR1 based polytope vaccine candidate (Torresi et al 2006).

We further checked the gene expression profile for mice immunized with various antigen formulations either with or without adjuvants using Taqman Low Density Array (TLDA). The adjuvants, when given alone without antigens, did not show significant change in gene profiling when compared with PBS control, thereby indicating that the immune response of the antigen was enhanced / modified because of the adjuvanted antigens. The adjuvants (Al(OH)3 / cadB) by themselves did not cause alteration in gene regulation.

On comparing HVR1 variant pool without adjuvant and HVR1 variant pool given either with cadB or aluminium hydroxide, no significant difference in the gene profile was recorded. Both Th1 and Th2 genes were up regulated indicating a balanced immune response in HVR1 pool-pNS3-cadB group, as compared to
the non-adjuvanted HVR1 pool-pNS3 group (figure52, table28). This indicated
the role of liposome encapsulation in upregulation of cytokine levels. Thus the
observed gene profile could be attributed the additive effect of NS3, cadB and
HVR1 pool.

A similar phenomenon was not observed with the tetramer-NS3
combinations, indicating that individual peptides rather than the tetramer could
generate better cytokine response, despite similar antibody titres produced
against HVR1 variant pool and HVR1 tetramer (figures44,45). This further
strengthens our conclusion that HVR1 variant pool, and not HVR1 tetramer
should be used for further studies.

The HVR1 pool-NS3-cadB combination induced optimum antibody titres
against both the HVR1 and NS3 components. A Th1/Th2 balanced isotyping
profile was also observed for both the candidate vaccine components. Though
the CBA results were inconclusive, the TLDA data strongly supported the efficacy
of this combination in comparison to the HVR1 tetramer-NS3 combinations
tested. Moreover, the HVR1 variant pool showed 72.2% reactivity with anti-HCV
positive human sera tested by ELISA. Overall, it was seen that the HVR1 pool-
NS3 combination encapsulated in cadB was the most promising vaccine
candidate.

In conclusion, the combination of peptides of HVR1 consensus sequence
and its variants along with pNS3 encapsulated in liposomes could generate both
cellular and humoral immune responses in mice. This preparation deserves
further evaluation in non-human primates/ humanized mouse models. We hope
that our work will prove useful in the development of genotype 3-based hepatitis
C vaccine.