Immunogenicity and antigenicity of the ATPase/helicase domain of the hepatitis C virus non-structural 3 protein

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The immunogenicity and antigenicity of an enzymatically functional (ATPase/helicase) recombinant protein encompassing residues 1207-1612 of the hepatitis C virus (HCV) non-structural 3 (NS3) protein was characterized using B10 congenic mice. Previous studies have indicated a high frequency of NS3-specific antibodies in HCV-infected humans. Similarly, all six immunized murine haplotypes were antibody responders to the NS3 ATPase/helicase domain, with the H-2k and H-21 haplotypes as high responders. As also observed in HCV-infected humans, the murine NS3 antibodies were predominantly directed to conformational determinants. Irrespective of the murine haplotype, IgG1 predominated in the primary anti-NS3 response, whereas IgG1 and IgG2b predominated in the secondary response. The antibody responder hierarchy was reiterated at the T cell level, with the H-2k and the H-21 haplotypes as the best responders. In both the H-2d and H-2k haplotypes ATPase/helicase-primed T cells secreted interleukin 2 and interferon γ, corroborating observations from HCV-infected humans. In the H-2d, H-2k and H-21 haplotypes the fine specificity of the T cell recognition of the ATPase/helicase domain was further characterized. Multiple, although generally weak, T cell recognition sites were found for all three haplotypes. The large size of the NS3 protein together with the presence of multiple class II binding motifs explain the high prevalence of NS3 antibodies in immunized mice and predict a similar explanation for the observed high frequency of NS3-specific antibodies in HCV-infected humans.

Introduction

The non-structural 3 (NS3) protein of hepatitis C virus (HCV) has been found to have several different functions. The N-terminal third of NS3 contains a serine protease domain which, alone or together with NS4a, cleaves the precursor polyprotein downstream of the NS3–NS4a junction (Barten-schlager et al., 1993; Grakoui et al., 1993a, b). The C-terminal two thirds of the NS3 sequence has been found to contain domains for both ATPase and helicase activity (Jin et al., 1995). Possibly owing to these vital enzymatic functions, the NS3 protein is one of the HCV proteins with the lowest sequence variability with respect to virus genotypes. Therefore NS3 may be a valuable target for the design of both enzymatic and immunological therapeutics.

In the HCV-infected human host the NS3 protein seems to be fairly immunogenic. This is supported by several observations. First, a majority of patients with chronic HCV infection develop antibodies to NS3 (Lok et al., 1993). Secondly, early in the acute phase of infection NS3 antibodies appear to be the most prevalent, as determined by recombinant proteins (Diepolder et al., 1995). Thirdly, in vitro T cell activation in response to NS3, although generally weak, has been detected in a number of studies of patients with acute or chronic HCV infection (Diepolder et al., 1995; Ferrari et al., 1994; Hoffmann et al., 1995). It was recently proposed that a strong in vitro T cell reaction to NS3 correlated with clearance of acute HCV infection whereas a less vigorous, or absent, NS3-specific T cell reactivity was observed in those who progressed to chronicity.
(Diepolder et al., 1995). It appears somewhat inconsistent that despite a seemingly strong NS3-specific T cell activity in the acute phase, significant NS3 antibody responses do not develop until progression to chronicity when the T cell activity is low.

To characterize the immunogenicity and antigenicity of the ATPase/helicase domain of HCV NS3, we immunized inbred mice with an enzymatically functional recombinant protein. We found that the recombinant NS3 protein is quite immunogenic in mice and some aspects of the murine immune response are similar to observations in HCV-infected humans with respect to both B and T cells.

Methods

**Mice.** Inbred B10 mice, B10 (H-2^b^), B10.D2 (H-2^d^), B10.M (H-2^f^), B10.BR (H-2^g^), B10.P (H-2^p^) and B10.S (H-2^s^), and BALB/c (H-2^b^) mice were obtained from either the breeding facility at The Scripps Research Institute or through BK Universal. All mice were used at 4–10 weeks of age.

**Recombinant NS3 ATPase/helicase domain protein.** The expression, purification and enzymatic characterization of a recombinant HCV NS3 (rNS3) protein, genotype 1a, covering residues 1207–1612 has previously been described (Jin et al., 1995). Prior to use as a recall antigen in T cell cultures, rNS3 was dialysed against PBS overnight and sterilized by UV-irradiation for 20 min.

**Synthetic peptide analogues.** The synthesis of overlapping peptides corresponding to the complete sequence of HCV NS3 of genotype 1a (Takanizawa et al., 1991) has been described (Zhang et al., 1994). Additional peptides were produced by a multiple peptide synthesizer (Syro MultiSynTech; Syntex) using Fmoc-protected amino acids. The peptides were cleaved and deprotected according to standard protocols for Fmoc peptide synthesis (Sallberg et al., 1991). The crude peptides were analysed by reversed phase HPLC using a PepS-5 C18 column (Pharmacia).

**ELISA.** All ELISAs for the detection of murine anti-NS3 antibodies were essentially performed as described previously (Milich et al., 1987). In brief, rNS3 was passively adsorbed at 0.4 µg/ml into 96-well microtitre plates in 50 mM-sodium carbonate buffer pH 9.6, overnight at 4 °C. Serial dilutions of sera were then incubated on the plates for 90 min. Bound murine serum antibodies were detected by either rabbit anti-mouse IgG (Sigma), or rabbit anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Collaborative Biomedical) followed by a peroxidase-labelled goat anti-rabbit IgG (Boehringer Mannheim; Milich et al., 1995).

Analysis of reactivities to linear determinants was performed using the NS3-specific synthetic peptides coated onto microtitre plates at a concentration of 10 µg/ml. The remainder of the assay was performed as described above.

**T cell proliferation and cytokine assays.** Groups of three to five mice were injected with 50 µg of rNS3 emulsified in Freund's complete adjuvant either intraperitonitely (i.p.) or subcutaneously (s.c.) in the hind footpads or at the base of the tail. Nine to 11 days later the mice were sacrificed and the spleens or draining lymph nodes were harvested. Single cell suspensions were prepared in Click's media and plated on microtiter plates at 6 x 10^5 cells per well for proliferation assays and 8 x 10^4 cells per well for cytokine assays. Supernatants (100 µl) were removed at 24 h for determination of interleukin 2 (IL-2) and at 48 h for determination of IL-4 and interferon γ (IFN-γ). The plates for T cell proliferation were incubated for 96 h with the addition of 1 µCi of [3H]thymidine (Tdr; Amersham) for the last 16 h. The labelled cells were harvested onto cellulose filters, quenched and the level of [3H]thymidine incorporation was determined by a liquid scintillation p-counter.

The presence of cytokines was determined as previously described (Milich et al., 1995). In brief, the presence of IL-2 in supernatants was determined by proliferation of the IL-2-sensitive NKA cell line. The presence of IL-4 was determined by the IL-4-sensitive CT4S cell line and the presence of IFN-γ was determined by a sandwich ELISA (Milich et al., 1995).

Results

**Influence of MHC genes on murine immune recognition of the ATPase/helicase domain of NS3**

Groups of three to six H-2 congenic B10 mice, haplotypes H-2^b^, H-2^d^, H-2^f^, H-2^p^ and H-2^a^, were primed and boosted (four weeks later) i.p. with 20 µg of rNS3 in adjuvant. Within the six haplotypes two were high responders (H-2^a^ and H-2^b^), three were intermediate (H-2^d^, H-2^f^ and H-2^p^) and one was a low responder (H-2^a^; Fig. 1). This confirms the influence of the murine MHC genes on the ability to respond to rNS3. The serum antibody end-point titres were within the same range as those observed to the ATPase/helicase domain in HCV-positive human sera (titre range 1:2500 to 1:100000; M. Chen, M. Sallberg, A. Sonnerborg, L. Jin, A. Birkett, D. Peterson & D. R. Milich, unpublished results). The primary NS3 response in the two high responder strains was restricted to IgG1, whereas the secondary response in all strains was predominantly IgG1 and IgG2b (Fig. 1). In the H-2^b^, H-2^f^ and H-2^p^ haplotypes 10- to 100-fold lower levels of IgG2a and/or IgG3 were also detected. The H-2^f^ haplotype showed the lowest total anti-NS3 IgG as well as the lowest IgG1 and IgG2b titres, which suggested a low responder status. Interestingly, the IgG isotype pattern of the primary response may have a counterpart in the human NS3-specific IgG subclass response, which is highly restricted (M. Chen, M. Sallberg, A. Sonnerborg, L. Jin, A. Birkett, D. Peterson & D. R. Milich, unpublished results).

**Specificity of the murine B cell response to the NS3 ATPase/helicase domain**

In contrast to other HCV proteins such as the core and NS4 proteins, there is a less frequent and lower level of antibody reactivity to linear NS3 B cell determinants represented by synthetic peptides in most human sera (Khudyakov et al., 1995; Sallberg et al., 1992, 1993; Zhang et al., 1995). This was also true in NS3 ATPase/helicase-immunized mice. Low level antibody reactivity to linear determinants was observed in the H-2^b^ and H-2^f^ haplotypes and less frequently or not at all in the other four haplotypes (Fig. 2 and data not shown). Reactivities to these regions are also rare in sera from HCV-infected patients (Khudyakov et al., 1995; Zhang et al., 1994). Also, murine antibody titres to linear determinants were at least 10-fold lower compared to the rNS3 protein, confirming that the reactivity was predominantly directed against a...
Fig. 1. Humoral responses to rNS3 in B10 congenic mice of six haplotypes. (a) Mice were injected at week zero and week 2 with 20 μg of rNS3 in Freund's complete adjuvant; the two bars represent bleeds at week 2 (1°) and week 6 (2°). All sera were tested at six fourfold dilutions starting at 1:60. Each value represents the endpoint titres of pooled sera from three mice. A reaction was considered positive if it exceeded the absorbance of non-immunized mice by three times. Isotypes of anti-NS3 antibodies at two (b) and six (c) weeks following first immunization. Each value represents the endpoint titre of pooled sera from a group of three mice. Endpoint titres were determined as described for total IgG.

Fig. 2. Mapping of reactivities in the 4 week sera from pooled B10.D2 (H-2^d) and B10.BR (H-2^k) mice to linear NS3 determinants represented by 16 amino acid peptides with a six amino acid overlap (a, b). The titres of the identified linear reactivities in both haplotypes were compared to those to rNS3 (c, d). Values are given as A_{492} values.
discontinuous or conformationally dependent determinant(s) in both haplotypes. This was true irrespective of the isotype of IgG (Fig. 2c, d).

Specificity of the murine T cell response to the NS3 ATPase/helicase domain

When analysing in vivo-primed rNS3-specific T cells by in vitro recall, the antibody responder hierarchy was reiterated, with the H-2^k and H-2^a haplotypes as the high responders and a weaker response in the H-2^b and H-2^d haplotypes (Fig. 3a). The H-2^f and H-2^p haplotypes were not further evaluated at the T cell level.

To characterize further the phenotype of polyclonal H-2^a- and H-2^k-restricted T cells specific for the NS3 ATPase/helicase domain, groups of two to three mice (BALB/c and C3H/He) were primed with 50 µg of rNS3 in the hind footpads. The phenotypes of the polyclonal responses were analysed with respect to secretion of IL-2, IL-4 and IFN-γ. Irrespective of the H-2 haplotype, ATPase/helicase-primed T cells predominantly secreted IL-2 and IFN-γ into culture supernatants (Fig. 3c, d). Low levels of IL-4 could only be detected in the H-2^k supernatants (Fig. 3e). Thus, the phenotypes of the polyclonal murine T cell responses to the ATPase/helicase domain of NS3 in the H-2^a and H-2^k haplotypes were Th1- or

Fig. 3. Comparison of rNS3-specific T cell priming efficiency in mice of four haplotypes (b, d, k and s) by s.c. injections in the hind footpads of 20 µg rNS3 in Freund’s complete adjuvant. Groups of three mice were primed and sacrificed 9 to 11 days later. Draining lymph nodes were harvested and single cell suspensions were cultured for 24 h in the absence or presence of varying amounts of dialysed rNS3. Culture supernatants were then removed and analysed for the presence of IL-2 (a) using the IL-2-sensitive NKA cell line. Values are given as c.p.m., with antigen minus the mean c.p.m. of wells without antigen (c.p.m.). Also given are the cytokine profiles of BALB/c (H-2^d; b) and B10.BR (H-2^k; c) lymph node T cells primed as described above and recalled by rNS3 in vitro. The cytokine assays (b, c, d) were performed as described in Methods. Values have been converted to units/ml using a dilution series of the respective cytokine.
Th0-like. This is similar to reports of human NS3-specific T cell clones (Diepolder et al., 1995).

The fine specificity and characteristics of the ATPase/helicase-specific T cell response were studied in the H-2d, H-2k and H-2s haplotypes. Mice were primed with 50 μg of rNS3 in complete adjuvant given i.p. or in the hind footpads, and 9 to 11 days later spleens or draining lymph nodes were harvested and tested for in vitro recall responses using rNS3 and overlapping peptides. All three haplotypes recognized multiple determinants within the 400 amino acid sequence (Fig. 4 and data not shown). The approximate locations of H-2d T cell recognition sites were at residues 1307-1322, 1367-1382 and 1597-1612; for H-2k mice residues 1237-1252, 1387-1402, 1447-1462 and 1547-1562 were recognized, and for H-2s mice 1217-1232, 1307-1322 and 1517-1532 were recognized (Fig. 4 and data not shown). Given the length of this protein, the presence of multiple, although weak, MHC-restricted T cell recognition sites in high responder murine strains to NS3 is not unexpected. The ability of the four identified H-2k-restricted T cell sites to prime ATPase/helicase-specific T cells was analysed by priming groups of B10.BR and C3H/He mice with 100 μg of the respective peptide or with 25 μg rNS3 as a control. The primed T cells were then analysed in vitro for the ability to be recalled by peptide or rNS3. All synthetic T cell sites were able to prime T cells which were recalled by recombinant ATPase/helicase protein in vitro and vice versa (Fig. 4 and data not shown). This verifies that these sites are also generated through antigen-presenting cell processing of rNS3 as determined by both proliferation (Fig. 4 and data not shown) and cytokine production (data not shown) in vitro. However, in two cases (1237-1252 and 1547-1562) the peptide-primed T cells were more efficiently recalled by the immunogen itself than rNS3 (data not shown) suggesting that further analysis of N- and C-terminally truncated analogues of...
Table 1. Alignment of HCV NS3 genotype-specific sequences within the identified H-2k-restricted T cell determinants

A previously mapped chimpanzee CTL epitope at residues 1447-1554 has been underlined (Erickson et al., 1993; Weiner et al., 1995).

<table>
<thead>
<tr>
<th>Strain/accession number*</th>
<th>HCV genotype</th>
<th>Location and sequence within HCV NS3</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1237-1252</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1447-1462</td>
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<td></td>
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<tr>
<td>HCV-1</td>
<td>1a</td>
<td>STKVPAAYAAGYKVL</td>
</tr>
<tr>
<td>HCV-H</td>
<td>1a</td>
<td>---K</td>
</tr>
<tr>
<td>BK</td>
<td>1b</td>
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<tr>
<td>HCV-J</td>
<td>1b</td>
<td>V</td>
</tr>
<tr>
<td>HC-J6</td>
<td>2a</td>
<td>S</td>
</tr>
<tr>
<td>HC-J8</td>
<td>2b</td>
<td>S-V-G--T--</td>
</tr>
<tr>
<td>1183033</td>
<td>2e/f</td>
<td>S-V-G--T--</td>
</tr>
<tr>
<td>633202</td>
<td>3a</td>
<td>V-G--M--</td>
</tr>
<tr>
<td>1183029</td>
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<td>V-G--M--</td>
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the respective regions is needed to define precisely the T cell site boundaries.

The identified approximate T cell sites in the H-2k haplotype were further analysed with respect to genotypic sequence variability to determine whether the genotype of HCV NS3 might influence the response. Alignments of three out of the four T cell sites are listed in Table 1. The fourth site at residues 1387-1402 did not show any variability within genotypes 1a, 1b, 2a, 2b, 2e/f or 3a. All other sites showed a varying degree of genotypic sequence variability especially with respect to genotype 2 (Table 1). Taken together this would suggest that genotype-specific and non-cross-reactive NS3-specific T cells may be elicited. However, using the H-2k haplotype as an example, a non-responder status due to genotypic variations can be excluded since one site was completely conserved. This indicates that the HCV NS3 protein may also induce cross-reactive T cell responses in HCV-infected humans.

Discussion

Apart from the defined enzymatic functions of the HCV NS3 protein in the virus life-cycle little is known about the intrinsic immunogenicity and antigenicity of NS3 in HCV-infected humans. Most patients with chronic HCV infection develop antibodies to NS3 and some demonstrate detectable NS3-specific T cells in vitro (Ferrari et al., 1994; Hoffmann et al., 1995; Iwata et al., 1995; Lok et al., 1995). In contrast, HCV-infected patients with acute infection appear to have readily detectable NS3-specific T cells but less frequently produce NS3-specific antibodies (Diepolder et al., 1995). These observations suggest several possibilities. Either the HCV NS3 protein is an intrinsically poor immunogen in humans or the presence of NS3 antigen is low throughout the infection, thereby limiting NS3-specific immune responses. To investigate the immunogenicity of the ATPase/helicase domain of NS3 we immunized inbred mice of different haplotypes and analysed the humoral and cellular responses. We found that the level of the response to the NS3 ATPase/helicase domain was MHC-dependent. However, none of the six haplotypes was found to be a non-responder to NS3. Moreover, the humoral responder status was dependent on the T cell responder status since both results correlated very well. In three haplotypes we observed multiple T cell recognition sites within NS3. In combination with the 400 plus amino acid length of the ATPase/helicase domain of NS3 this explains the high frequency of NS3 antibodies in mice. Most likely this is also true in humans since T cell nonresponsiveness to NS3 in an outbred human population is even less likely. Thus, no data obtained in the murine model of the immunogenicity of the HCV NS3 ATPase/helicase domain would suggest an intrinsically unusual or poor immunogenicity for this protein. Additionally, no data to date suggest that the N-terminal protease domain interferes with the immunogenicity or antigenicity of the ATPase/helicase domain (M. Chen, M. Sällberg, A. Sonnerborg, L. Jin, A. Burkett, D. Peterson & D. R. Milich, unpublished results). Therefore, the seemingly limited immunogenicity of HCV NS3 in humans may be secondary to low levels of NS3 protein expression during both acute and chronic phase HCV infections.

Interestingly, the majority (7/10) of the identified murine T cell sites coincide with a possibly dominant region for human Th cell recognition of NS3 spanning the residues 1207-1488 (Diepolder et al., 1995). Also, the identified H-2k-restricted NS3-specific T cell site at residues 1447-1462 completely overlapped a previously identified cytotoxic T cell (CTL) epitope recognized by a chimpanzee with chronic HCV infection (Erickson et al., 1993). A recent paper from the same
group suggested the emergence of a CTL escape mutation within this site (Weiner et al., 1995).

Overall the human and murine immune responses to the NS3 ATPase/helicase domain are similar. Both species seem to develop antibodies which primarily recognize discontinuous or conformationally dependent determinants within HCV NS3. However, peptidic NS3-specific reactivities of a much lower magnitude can also be found in both hosts (Khudyakov et al., 1995; Zhang et al., 1994). This is in contrast to the human and murine serological responses to the HCV core and NS4 proteins in which reactivities directed towards linear determinants are frequently observed (Kakimi et al., 1995; Khudyakov et al., 1995; Simmonds et al., 1993; Sällberg et al., 1992, 1993; Zhang et al., 1995).

Also, with respect to the phenotype of the primed NS3-specific T cells, similarities can be found between mice and humans. Both the murine primary antibody response and the NS3-specific antibody response in chronically infected humans are restricted to a single IgG isotype (M. Chen, M. Sällberg, A. Sonnerborg, L. Jin, A. Burkett, D. Peterson & D. R. Milich, unpublished results). Moreover, irrespective of the species and the MHC of the host, the phenotype of the NS3-specific T cell response seems to be biased towards IL-2 and IFN-γ production (Diepolder et al., 1995). Whether this has any significance to HCV infections remains to be determined. In mice the predominance of anti-NS3 IgG1 in the primary response (positively regulated by Th2 cytokines) and detection of mainly Th0/Th1 cytokines in culture supernatants may seem inconsistent. This may, however, accurately reflect the nature of a polyclonal response, as indicated by the mixtures of IgG isotypes developed in the secondary response. We are currently studying this further.

The human and murine immune responses to the ATPase/helicase domain of HCV NS3 show a number of similar characteristics. The presence of multiple NS3-derived MHC class II binding motifs explains the high frequency of NS3-specific antibody production in mice and may also serve to explain the high prevalence of anti-NS3 antibodies in HCV-infected imunocompetent humans.

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