Immune responses to the hepatitis C virus NS4A protein are profoundly influenced by the combination of the viral genotype and the host major histocompatibility complex

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The interaction between the host major histocompatibility complex (MHC) and the genotype of the hepatitis C virus (HCV) was analysed using synthetic full-length non-structural (NS) 4A proteins, residues 1658–1712, of genotypes 1b, 2b, 3a, 4a and 5a. Human and murine antibodies specific for the five NS4A genotypes analysed focused on residues 1688–1707. In immunized B10 H-2 congenic mice, the H-2d, H-2f and H-2s haplotypes were good responders to NS4A, irrespective of the viral genotype. In contrast, the H-2k haplotype was a low or non-responder to all NS4A genotypes, except for genotype 2b. Also, H-2k- and H-2f-restricted NS4A genotype 1b-specific T-cells focused on residues 1670–1679 and 1683–1692, respectively, whereas H-2k-restricted genotype 2b-specific T-cells focused on the carboxy terminus. Interestingly, H-2f-restricted genotype 1b-specific T-cells did not cross-react with T-cell site analogues of seven other genotypes, whereas the H-2k-restricted, genotype 1b-specific T-cells cross-reacted with genotypes 1a, 4a and 5a. Thus the combination of viral genotype and host MHC profoundly influences the ability to mount an HCV NS4A-specific immune response.

Introduction

One major factor adding to the high rate of chronic infections caused by the hepatitis C virus (HCV) may be the high degree of variability between different genotypes of HCV (Bukh et al., 1993, 1994), and the different quasi-species which can be found within one infected host (Hijikata et al., 1991; Weiner et al., 1991). With respect to the B-cell recognition of HCV proteins, it has been recognized that genotype- and strain-specific antibodies are produced within the infected host (Kato et al., 1993, 1994; Weiner et al., 1992). Even to the most conserved protein of HCV, the structural core protein, genotype-specific antibodies can be detected using synthetic peptides (Machida et al., 1992; Zhang et al., 1994). However, most of the human serology of HCV has so far been conducted using truncated recombinant proteins, recombinant fusion protein, or short synthetic peptides. Information on the complete antibody response to full-length and conformationally correct HCV proteins is limited due to the lack of native or native-like recombinant proteins.

To address some of these issues, a murine model was developed wherein a short protein with a high degree of variability with respect to HCV genotype may be analysed. The advantage of such a system is that it minimizes the number of variables that may influence the humoral and cellular responses (i.e. the presence of multiple B- and T-cell recognition sites). Furthermore, a short protein such as the non-structural (NS) 4A can be readily produced in several genotype- and strain-specific variants using controlled chemical synthesis. Previous serological experiences with the C5-1-1 protein, which contains a part of NS4A (Kuo et al., 1989), and more recent synthetic peptide-based data clearly imply that human B-cell responses are highly genotype-specific (Bhattacharyee et al., 1995; Simmonds et al., 1993; Zhang et al., 1994). It may be assumed that, similar to human B-cell recognition of NS4A, human T-cell recognition may be highly dependent on the HCV genotype.
NS4A was recently shown to perform vital functions in the life cycle of the HCV virus. During processing of the HCV polyprotein the protease domains of NS2 and NS3 cleave the NS3/NS4A junction, after which NS4A is complexed to the amino-terminal serine protease domain of NS3 (Bartenschlager et al., 1993; Grakoui et al., 1993a, b, c). The binding to NS3 has been found to be dependent on residues 1675–1686 of NS4A (Bartenschlager et al., 1995; Shimizu et al., 1996). Recent studies have shown that the cleavage of the precursor protein at the NS3/NS4A, NS4A/NS4B and NS4B/NS5A junctions is dependent on NS4A whereas the cleavage of the NS5A/NS5B is less dependent on, or independent of, the presence of NS4A (Bartenschlager et al., 1995; Satoh et al., 1995).

Present data have shown that, unlike chronic hepatitis B virus infections, in the chronic phase of HCV infection HCV-specific CD4+ T-cell activity (Botrell et al., 1993; Diepolder et al., 1995; Ferrari et al., 1994; Hoffmann et al., 1995; Koskinas et al., 1994) and CD8+ T-cell activity (Erickson et al., 1993; Koziel et al., 1993, 1995) can be readily detected. Despite the presence of an active T-cell population, the magnitude of the humoral responses, such as those to the core protein, seems to be significantly lower in chronic HCV infection than in chronic HBV infection (Maruyama et al., 1993a, b; Sällberg et al., 1989; Zhang et al., 1995). The reasons for this are poorly understood but could be due to intrinsic differences in the immunogenicity of HBV- and HCV-encoded proteins. Other factors may be how the different proteins are perceived and processed by the immune system, the types of T-cell populations that are induced, or the amount of antigens available. With respect to human T-cell recognition of different HCV proteins, mainly Th1- or Th0-like cytokines such as IL-2, IL-4 and γIFN have so far been observed in T-cell clones, in T-cell lines, or in polyclonal T-cell responses against the HCV core, NS3, and NS4 proteins (Diepolder et al., 1995; IWata et al., 1995; Minutello et al., 1993).

Murine responses following immunization with HCV proteins have not yet been extensively studied. Most reports have focused on the murine recognition of truncated recombinant versions of the HCV core protein (Chen et al., 1995; Harase et al., 1995; Kakimi et al., 1995) or naked DNA constructs containing the core gene (Lagging et al., 1995; Major et al., 1995). Interestingly, endogenously produced HCV core protein does not seem very immunogenic in mice (Lagging et al., 1995; Major et al., 1995). We recently showed that HCV NS3-specific immune responses in immunized mice seem to be insensitive to influences from the viral genotype and the MHC of the host (Sällberg et al., 1996). This is most probably due to the substantial length of the NS3 protein.

Based on previous observations we produced a full-length HCV NS4A protein of genotype 1b and characterized the human and murine humoral responses as well as the murine T-cell response. We found that, depending on the MHC of the responding host, NS4A-specific T-cells may be highly genotype-specific.

**Methods**

- **Human serum samples.** A total of 42 serum samples was obtained from patients with chronic HCV infections in which the HCV genotype was determined as described previously (Zhang et al., 1995).
- **Mice.** Inbred H-2 congenic B10 mice B10.D2 (H-2d), B10.M (H-2^d), B10.BR (H-2^b) and B10.S (H-2^s) were obtained either from the breeding facility at the Scripps Research Institute or from BK Universal, Sollentuna, Sweden. Additional BALB/c (H-2^d), C57/He (H-2^b) and CBA (H-2^k) mice were obtained from the same sources. All mice were used at 6–12 weeks of age.
- **Synthetic NS4A and shorter peptide analogues.** Five full-length versions of the HCV NS4A protein, residues 1658–1712, of genotypes 1b, 2b, 3a, 4a and 5a (Fig. 1; Bhattacharjee et al., 1995; Simmonds et al., 1993) were chemically synthesized by a continuous flow peptide synthesizer (Milligen 9050 Plus, Millipore). The synthesis was run using 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids under enhanced monitoring using quinoline yellow, with the allowed coupling efficacy set to >99.5%. Subsequent amino acid coupling was not initiated until that level had been reached. Between each coupling cycle an acetylation reaction was performed to terminate peptides with insufficient coupling. Finally, an amino-terminal tag of six histidine residues was added to the sequence to facilitate purification. The peptide was cleaved and deprotected according to standard protocols for Fmoc peptide synthesis (Sällberg et al., 1991). The crude peptide was first purified by reversed-phase high-performance liquid chromatography (RP–HPLC) using a PepS-15 C18 column (Pharmacia). The HPLC-purified fractions were then lyophilized and further purified over a chelating column using the histidine tag. The molecular mass of the obtained protein was determined both by SDS–PAGE sizing electrophoresis and by Western blotting using standard protocols (Phast System, Pharmacia) and was found to be the expected 6 kDa (data not shown). Molecular mass analysis by mass spectrometry (MALDI–TOF and PD) was inconclusive despite repeated attempts (data not shown). However, the integrity of the sequence was further checked by protein sequence analysis of the amino-terminal 16 residues by an independent laboratory (Department of Medical Biochemistry, Karolinska Institutet, Sweden). The obtained sequence (HHHHHHTWTVLVGGLV) corresponded perfectly to the amino-terminal sequence of NS4A genotype 1b. Moreover, the amino acid composition was analysed by total hydrolysis and showed an almost perfect correlation to the expected amino acid proportions of the full-length NS4A genotype 1b peptide.

Additional overlapping peptides corresponding to HCV NS4A of genotype 1b and other genotypes were produced by a multiple peptide synthesizer using Fmoc chemistry (Syro, Syntex, Germany). The peptides were analysed by RP–HPLC and the molecular masses were confirmed by MALDI–TOF mass spectrometry. All molecular masses obtained corresponded to the expected values. The results of some of these determinations are given in the text.

- **Enzyme immunoassays.** All enzyme immunoassays (EIAs) for the detection of human and murine anti-NS4A antibodies were performed essentially as described previously (Milich et al., 1987; Sällberg et al., 1992). In brief, NS4A peptides were coated at 10 µg/ml to 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 60 min. Bound murine serum antibodies were indicated by either rabbit anti-mouse IgG (Sigma) or rabbit anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Sigma) followed by a peroxidase-labelled goat anti-rabbit IgG (Milich et al., 1995). Human antibodies were detected by reagents previously described in detail (Sällberg et al., 1993).

- **T-cell proliferation and cytokine assays.** Groups of two to four
mice were injected with 100 μg NS4A emulsified in Freund’s complete adjuvant (CFA) intraperitoneally (i.p.) or subcutaneously (s.c.) in the hind foot pads or at the base of the tail. At 9–11 days later the mice were sacrificed and the spleens or draining lymph nodes were harvested. Single-cell suspensions were prepared in Click’s medium and plated in microplates at 6 × 10^6 cells per well for proliferation assays, and at 8 × 10^6 cells per well for cytokine assays (Milich et al., 1987, 1995). Supernatants were removed at 24 h for determination of IL-2, and at 48 h for determination of IL-4 and γIFN. The plates for T-cell proliferation were incubated for 96 h with the addition of 1 μCi [³H]thymidine (TdR; Amersham) for the last 16 h. The labelled cells were harvested onto cellulose filters, quenched, and the level of [³H]TdR incorporation was determined by a liquid scintillation β-counter.

The presence of cytokines was determined as previously described (Milich et al., 1995). Briefly, the presence of IL-2 in supernatants was determined by proliferation of the IL-2-sensitive NK'A cell line. The presence of IL-4 was determined by proliferation of the IL-4-sensitive CT.4S cell line, and presence of γIFN was determined by a sandwich EIA (Pharmingen).

**RT–PCR to detect cytokine mRNA.** Detection of cytokine mRNA (IL-2, IL-4, IL-10 and γIFN) and β-actin expression was performed by a RT–PCR. In brief, 2 × 10^6 cells were cultured in the absence or presence of 5 μg NS4A for 36 h in 1 ml Click’s medium. From the total RNA, 1 μg was extracted using TRIZol Reagent (Gibco), transcribed to cDNA using M–MLV reverse transcriptase (Gibco) and oligo(dT)_{12-18} according to the manufacturer’s recommendation. The cDNA was amplified with Taq polymerase (Promega) using primers designed to amplify indicated cytokine mRNAs (Stratagene). The presence of an amplicon was determined by gel electrophoresis and ethidium bromide staining.

**Results**

**Human antibody recognition of the NS4A protein.**

As an NS4A protein of native length has not previously been assayed with sera from HCV-infected patients, it was necessary to characterize its antigenicity. Sera were obtained from 10 HCV-infected patients of genotype 1a/1b (as determined by PCR genotyping) which were reactive in NS4 peptide-based serotyping described previously (Zhang et al., 1994). All 10 sera recognized the full-length NS4A genotype 1b protein at titres ranging from 1:100 to 1:24,300 (data not shown). Moreover, when analysing the NS4A-specific antibodies with respect to the IgG isotypes, the response was found to be restricted to IgG1, corroborating previous observations using short synthetic peptides (data not shown; Sällberg et al., 1993).
An additional 32 serum samples with HCV genotypes 1a/1b (n = 13), 2b (n = 13) and 3a (n = 6) were screened by EIA for NS4A antibodies using the corresponding 1b, 2b and 3a genotype-specific full-length NS4A peptides. Of the total 21 samples with NS4A antibodies (66%), nine were of genotype 1a/1b, eight of genotype 2b and four of genotype 3a.

Influence of MHC genes on the murine immune recognition of NS4A

Groups of three to four congenic B10 mice, haplotypes H-2d, H-2f, H-2k and H-2s, were primed and boosted 4 weeks later i.p. with 100 μg NS4A in adjuvant. Interestingly, within the four haplotypes two were high responders (H-2f and H-2s), one was a low responder (H-2d) and one was a non-responder (H-2k; Fig. 1) to NS4A genotype 1b. Using the other four genotypes of NS4A as immunogens indicated that H-2s was consistently a high humoral responder to NS4A. The H-2f haplotype was mostly a high responder to NS4A, with the exception of the response to genotype 2b, when H-2f was the lowest responder. However, none of the total 30 H-2f or H-2s mice immunized with the full-length NS4A peptides was a non-responder with respect to specific antibodies. In contrast, H-2d mice were consistently intermediate to low responders,
Genotype-specific immune response to HCV NS4A

**(a)** LN T-cell proliferation

![Graph showing LN T-cell proliferation](image)

- LN T-cell proliferation
- Amount NS4A genotype 1b (µg/ml)
- Amount HCV NS4A genotype 1b (µg/ml)
- LN [3H]TdR uptake (d.c.p.m.)
- CT4.S TdR uptake (d.c.p.m.)
- NK′A [3H]TdR uptake (d.c.p.m.)
- Amount NS4A genotype 1b (µg/ml)
- Draining lymph nodes were harvested and single-cell suspensions were cultured for 96 h in the absence or presence of varying amounts of NS4A.
- Values are given as counts per minute (c.p.m.) with antigen with subtraction of the mean c.p.m. of wells without antigen (δc.p.m.).
- Also given are the cytokine profiles of B10.M (H-2f) and B10.S (H-2s) lymph node T-cells, primed as described above and recalled by NS4A in vitro. The IL-2 (b), γIFN (c) and IL-4 (d) cytokine assays were performed as described in Methods. Peak values were also converted to units/ml using a dilution series of the respective cytokine. Values are given either as δc.p.m. or the δA<sub>492</sub>.

**Fig. 3.** Comparison of rNS4A-specific T-cell priming efficiency in four murine H-2 haplotypes (d, k, f and s) by s.c. injections in the hind foot pads of 100 µg NS4A in CFA (a). Groups of three mice were primed and sacrificed 9–11 days later. Draining lymph nodes were harvested and single-cell suspensions were cultured for 96 h in the absence or presence of varying amounts of NS4A. Values are given as counts per minute (c.p.m.) with antigen with subtraction of the mean c.p.m. of wells without antigen (δc.p.m.). Also given are the cytokine profiles of B10.M (H-2<sup>f</sup>) and B10.S (H-2<sup>s</sup>) lymph node T-cells, primed as described above and recalled by NS4A in vitro. The IL-2 (b), γIFN (c) and IL-4 (d) cytokine assays were performed as described in Methods. Peak values were also converted to units/ml using a dilution series of the respective cytokine. Values are given either as δc.p.m. or the δA<sub>492</sub>.

**Fig. 4.** Cytokine mRNA profiles of B10.M (<H-2<sup>f</sup>>) and B10.S (<H-2<sup>s</sup>>) lymph node T-cells primed as described in the legend to Fig. 3 and recalled by NS4A in vitro. The levels of β-actin (β-a), IL-2, γIFN and IL-4 mRNA were determined after 48 h culture in the absence (−) or presence (+) of 25 µg/ml NS4A using RT–PCR as described in Methods. The expected band sizes of the amplified cDNAs are 245 bp for β-actin, 451 bp for IL-2, 420 bp for IL-4 and 405 bp for γIFN. The molecular size marker is a φX-174 RF DNA HaeIII digest (Pharmacia).

whereas H-2<sup>k</sup> mice were in general non- or low responders to NS4A with the exception of genotype 2b (Fig. 1). These data confirm a profound influence of the murine MHC genes on the ability to respond to NS4A. Also, the data confirm that genotype 2 is the most divergent HCV strain from an immunological perspective.
The antibody titres in mice are similar to those observed in anti-NS4A-positive human sera. The NS4A-specific IgG isotype response was restricted to IgG1 in the primary responses in all H-2 haplotypes (data not shown). However, during the secondary responses most haplotypes developed significant, but 10- to 100-fold lower levels of IgG2a, IgG2b and IgG3 (data not shown).

**Fine specificity of the human and murine response to NS4A**

The human and murine antibody specificities were further mapped by short overlapping synthetic peptides, confirming that the major recognition site is located at the carboxy terminus, residues 1688–1707, as shown previously (Fig. 2; Simmonds et al., 1993; Sällberg et al., 1993; Zhang et al., 1994). No additional human or murine B-cell antigenic sites seem to reside outside the carboxy-terminal domain of NS4A genotype 1b. The murine B-cell response was found to overlap almost perfectly with the antibody response observed in HCV-infected humans, irrespective of the NS4A genotype used as the immunogen (Fig. 2 and data not shown).

The specificities of the antibodies induced by NS4A genotype 1b were studied in more detail. The NS4A genotype 1b-specific antibodies induced in the three responding haplotypes were overlapping and genotype-specific. All haplotypes were predominantly reactive to the NS4A peptide 1688–1707 corresponding to NS4A genotypes 1, 5a and 6a, and non-reactive to NS4A genotypes 2 and 3 (Fig. 2). However, human sera from patients infected with HCV genotype 1 also showed some or even high cross-reactivity to NS4A peptides 1688–1707 corresponding to genotype 4a.

To characterize further the molecular basis for the observed cross-reactivities of both human and murine recognition of NS4A, additional Ala or Gly substitution analogues of residues 1688–1707 of NS4A genotype 1b were produced. By analysing these peptides for recognition by the murine and human sera we found that the Arg at position 1698, Glu at position 1701 and Tyr at 1702 were highly
sensitive to substitutions in a majority of the sera tested (Fig. 2).

**Murine Th-cell response to NS4A**

When analysing in vitro primed NS4A genotype 1b-specific T-cells by in vitro recall, the responder hierarchy observed in antibody levels was perfectly reproduced, with the H-2^k^ and H-2^s^ haplotypes as the high responders and a weak T-cell response in the H-2^f^ haplotype (Fig. 3). Similarly, no in vitro NS4A-specific T-cell recall response could be observed in the H-2^k^ haplotype. Also, no difference was observed in the recall responses in the H-2^d^ and H-2^s^ haplotypes if mice of different non-H-2 genetic backgrounds were used (i.e. B10.D2 or BALB/c, and B10.JR or CBA) confirming that the low- or non-responder status resides in the H-2 locus (data not shown).

We further characterized the H-2^f^ and H-2^s^ haplotypes with respect to the Th-cell subset predominance of polyclonal NS4A-specific Th-cells. Groups of two to three mice were primed with 100 μg NS4A, either i.p. for the analysis of splenic T-cells, or in the hind foot pads for the analysis of NS4A-specific T-cells in the draining lymph nodes. Independent of the H-2 haplotype, NS4A-primed T-cells mainly secreted IL-2 and γIFN detected in culture supernatants from both lymph nodes and spleen (Fig. 3 and data not shown). Low-level IL-4 could be detected only in the H-2^f^ supernatants. However, with respect to cytokine mRNA expression, IL-2, IL-4 and γIFN mRNA could be detected independent of the haplotype and the analysed organ (Fig. 4 and data not shown). Since these cultures represent polyclonal responses the mixture of T-cell cytokines may well correspond to the mixture of observed IgG isotypes.

Human NS4A-specific T-cell responses from HCV-infected patients have not yet been studied. However, several studies have described human T-cells that recognize the C-100 protein, which contains mainly NS4B and only the carboxy-terminal part of NS4A (Simmonds et al., 1993; Sällberg et al., 1993; Zhang et al., 1994). In two recent studies it was found that cloned NS3- or NS4-specific liver-derived T-cells secreted IL-2, IL-4, IL-10 and γIFN, which is indicative of a Th0-like phenotype (Diepolder et al., 1995; Minutello et al., 1993).

**Fine specificity of murine Th-cell recognition of NS4A genotype 1b**

The fine specificity of the NS4A-specific T-cell response was characterized in the H-2^f^ and H-2^s^ haplotypes. Mice were primed with 100 μg NS4A i.p. or in the hind foot pads, and 9–11 days later spleens or draining lymph nodes were harvested and tested for in vitro recall responses using short overlapping peptides. Both haplotypes recognized distinct determinants towards the amino terminus from the B-cell recognition site (Fig. 5). The approximate locations were at residues 1668–1682 for the H-2^s^ haplotype and residues 1678–1692 for the H-2^k^ haplotype. Both these T-cell sites contain genotype-specific variations in the NS4A sequence.

The ability of the identified T-cell sites to prime NS4A-specific T-cells was analysed by priming groups of B10.M and B10.S mice with 100 μg of the respective peptides. The primed T-cells were then analysed in vitro for the ability to recognize NS4A. Both 20-amino-acid synthetic T-cell sites were found to prime NS4A-specific Th-cells since NS4A was able to recall specific proliferative and cytokine (IL-2 and γIFN) responses in vitro (data not shown).

The H-2^f^ and H-2^s^-restricted Th-cell sites putatively mapped to residues 1668–1682 and 1678–1692, respectively, were more precisely defined using 12-amino-acid-long peptides with an 11-residue overlap between adjacent peptides. B10.M and B10.S mice were immunized i.p. with 100 μg NS4A genotype 1b and spleens were harvested 10 days later. The H-2^f^-restricted Th-cell site was fine-mapped to the sequence LAAYCLTTGS at residues 1670–1779, and the H-2^k^ restricted Th-cell site was fine-mapped to the sequence VGRIILSGRP at residues 1668–1682. Both peptides were further analyzed using B-cell site-specific T-cells, which are also able to recognize the two complexes. Both peptides were found to be recognized by both B10.M and B10.S mice, indicating that the H-2^f^ and H-2^s^ sites are both present in both haplotypes. The approximate locations were at residues 1668–1682 for the H-2^s^ haplotype and residues 1678–1692 for the H-2^k^ haplotype. Both these T-cell sites contain genotype-specific variations in the NS4A sequence.
residues 1683–1692 (Fig. 5). There was some variability between different experiments as to whether the amino-terminal Leu residue was necessary for H-2f-restricted Th-cell recognition (data not shown).

These results also show that the short H-2f- and H-2s-restricted T-cell peptides for which the molecular mass was confirmed by mass spectrometry (ALAAYCLTTGSV, \( M_r = 1169 \pm 5 \); VGRIILSGRPAI, \( M_r = 1250 \pm 4 \)) primed T-cells which were recalled in vitro by the full-length NS4A protein. Thus verified peptides corresponding to the amino termini and middle regions of the full-length NS4A protein, which may be the least reliable parts of NS4A due to the length of the synthesis, elicited T-cells which recognized full-length NS4A. Together with the amino-terminal sequencing of NS4A, this is strong, direct evidence that the full-length NS4A also contains these sequences, and further confirms the integrity of the obtained synthesis product.

The profound influence of the NS4A genotypes on the T-cell responder status of the H-2k haplotype mice was also studied. Groups of five C3H mice were primed with 100 µg NS4A genotypes 1b and 2b, respectively, in the hind foot pads. In vivo primed T-cells were then recalled by both genotype 1b and 2b NS4A. As predicted from the humoral studies, NS4A genotype 1b does not prime H-2k-restricted T-cells, and NS4A genotype 2b-primed T-cells do not cross-react with NS4A genotype 1b (Fig. 6). The recognition site for the H-2k-restricted NS4A genotype 2b-specific T-cells was mapped to the carboxy-terminal region 1688–1711, which contained numerous genotype-specific sequence variations (Fig. 6).

**Cross-reactivity of H-2f- and H-2s-restricted HCV NS4A genotype 1b-specific T-cells**

To evaluate the genotypic cross-reactivity of NS4A genotype 1b-primed T-cells, an additional set of synthetic analogues of the H-2f- and H-2s-restricted T-cell sites was produced, corresponding to genotypes 1a, 2a, 2b, 2c, 3a, 4a, 5a and 6a. These analogues were analysed for the ability to recall T-cells primed with the T-cell sites corresponding to genotype 1b. The H-2f-restricted genotype 1b-specific T-cells were highly specific and showed no cross-reactivity to other genotypes (Fig. 7). In contrast, H-2s-restricted genotype 1b-specific T-cells were cross-reactive with genotypes 1a, 4a and 5a (Fig. 7). No cross-reactivity was observed to genotypes 2a, 2b, 2c, 3a and 6a, which indicates that some of the genotypic variations contained within these peptides are important residues in the recognition of genotype 1b.
Different regions of NS4A show different degrees of sequence variability in terms of the genotypes of HCV. Therefore, the locations of immunological recognition sites would largely determine the ability of the host MHC to cross-react between different genotypes. As shown in Fig. 1, the locations of both human and murine B-cell recognition sites confirm that NS4A-specific antibodies should be less likely to cross-react with NS4A of genotype 2, as compared to the other genotypes. The lack of cross-reactivity in our NS4A genotype 1-specific murine and human sera to the genotype 3 peptide is most probably explained by the conservative Arg-to-Lys substitution at position 1698. Using the substitution peptide analogues we often found that this residue could not be substituted by Ala and retain antibody recognition (Fig. 1).

With respect to genotypic variations within the fine-mapped murine T-cell recognition sites, it is clear why genotype 1b-specific Th-cells may not be cross-reactive to the other genotypes of NS4A. The H-2^b-restricted Th-cell site is not reactive to NS4A of genotypes 2 and 3, most probably due to the extensive variability. However, even more subtle changes within the H-2^d-restricted T-cell site are sufficient to prevent cross-reactivity.

Discussion

Many facets of the immune response of the infected host to HCV proteins are unclear. Even though HCV-specific antibodies, CD4^+ Th-cells and CD8^+ CTL can be simultaneously found in an active infection, in the vast majority of cases the immune response is not successful in eradicating the HCV infection. Additionally, a selective immune pressure on HCV immune response is not successful in eradicating the HCV found in an active infection. In the vast majority of cases the results verify the profound influence of the MHC genotype of the host on the B-cell and T-cell immune response to NS4A. In H-2^k haplotype mice, neither NS4A, genotype 1b-specific antibodies nor T-cells could be detected. Furthermore, this low- or non-responder phenotype also exists for NS4A genotypes 3a, 4a and 5a in the context of the H-2^k haplotype. Whether this is true in HCV-infected humans remains to be investigated. We did note that H-2^k haplotype mice were good responders to NS4A genotype 2b. This was explained by the finding that the H-2^k-restricted T-cell site within NS4A genotype 2b was located to the carboy terminus, which contains a substantial amount of genotype-specific sequence variations. Since NS4A is extremely small, some human leucocyte antigen types may well be non-responders to NS4A.

However, if the human immune system recognizes NS4A as a complex with the substantially larger NS3 protein, NS3-specific Th-cells may provide help to circumvent NS4A Th-cell non-responsiveness. We recently found that none of six murine B10 haplotypes was non-responsive to HCV NS3, confirming that the length of this protein may prevent murine non-responsiveness to NS3 (Sällberg et al., 1996). The present data show that a murine non-responder haplotype to NS4A genotype 1b exists and, together with the present (66 %) and previously reported (42%; Sällberg et al., 1993) comparatively low frequency of NS4A antibodies in HCV-infected humans, may suggest that human NS4A-specific B-cells do not receive help from NS3-specific T-cells.

The phenotype of NS4A genotype 1b-specific murine T-cells appears similar to that of NS4-specific T-cells in humans. The NS4A-specific murine polyclonal T-cell response was mainly of a Th1/Th0 phenotype independent of MHC. Human NS4A/4b-specific T-cell clones have been found to correspond mainly to the Th0 phenotype (Minutello et al., 1993). Also, other HCV proteins such as the core and the NS3 proteins have been found to prime Th1/Th0-like IFN-securing T-cells in infected humans (Diepolder et al., 1995; Iwata et al., 1995).

In the H-2^d haplotype a genotype 1b-specific Th-cell recognition site was precisely defined to the sequence LAAAYCLTGTG at residues 1670–1679. Despite the comparatively limited genotypic variability within this region of NS4A, genotype 1b-primed H-2^d-restricted Th-cells were highly specific, with low cross-reactivity to the other genotypes of NS4A. In contrast, the H-2^d-restricted NS4A genotype 1b-specific response was relatively more cross-reactive. However, no cross-reactivity was observed to genotypes 2 and 3, which is explained by the fact that the 1678–1692 sequence contains numerous substitutions in these genotypes. These observations may be important in terms of the human HCV-specific T-cell response. As shown here, certain substitutions within a T-cell site are highly effective at avoiding recognition due to lack of cross-reactivity. Thus, mutations within human HCV-specific Th-cell recognition sites may be an effective way to hamper or even inactivate the Th-cell response, and subsequently also the B-cell response.
In both HBV and HCV, specific CD4+ cell responses have been detected during clinical remission and clearance of the infection (Diepolder et al., 1995; Jung et al., 1995). The lack of an efficient CD4+ response in the chronic phase of these two diseases may result from distinct causes. In an HBV infection, subviral proteins are excessively produced and secreted by the infected cell and a tolerant or anergic state may be induced in specific T-cells (Milich et al., 1990). This is most probably not the case during infections with HCV, in which the viral proteins can be detected only at low levels in a limited number of infected cells. The low levels of viral antigens during an ongoing HCV infection may also reflect the comparatively moderate magnitude of the humoral responses. During the acute symptomatic phase of HCV infection, specific antibody production is weak and does not develop until later stages of the disease after evolution to chronicity (Diepolder et al., 1995; Zhang et al., 1995). This may be secondary either to intrinsic immunological properties of the HCV proteins or to the fact that they simply are not produced in significant amounts. Moreover, the pronounced variability within HCV-encoded proteins may certainly hamper the effectiveness of the Th-cell priming event.

The murine model of NS4A recognition has some important features. Genotype-specific full-length versions of NS4A are relatively easy to produce and purify by standard techniques, allowing for detailed studies on the interaction between the genotype of the virus and that of the infected host. This model allows studies on how changes in the viral phenotype may influence the host response and possibly change the phenotype of the response (Windhagen et al., 1995). Moreover, we anticipate that it may be possible to perform similarly detailed studies on human T-cells using full-length NS4A sequences based on the virus infecting the human host.

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