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Naturally Occurring Antibodies That Recognize Linear Epitopes in the Amino Terminus of the Hepatitis C Virus E2 Protein Confer Noninterfering, Additive Neutralization

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Chronic hepatitis C virus (HCV) infection can persist even in the presence of a broadly neutralizing antibody response. Various mechanisms that underpin viral persistence have been proposed, and one of the most recently proposed mechanisms is the presence of interfering antibodies that negate neutralizing responses. Specifically, it has been proposed that antibodies targeting broadly neutralizing epitopes located within a region of E2 encompassing residues 412 to 423 can be inhibited by nonneutralizing antibodies binding to a less conserved region encompassing residues 434 to 446. To investigate this phenomenon, we characterized the neutralizing and inhibitory effects of human-derived affinity-purified immunoglobulin fractions and murine monoclonal antibodies and show that antibodies to both regions neutralize HCV pseudoparticle (HCVpp) and cell culture-infectious virus (HCVcc) infection albeit with different breadths and potencies. Epitope mapping revealed the presence of overlapping but distinct epitopes in both regions, which may explain the observed differences in neutralizing phenotypes. Crucially, we failed to demonstrate any inhibition between these two groups of antibodies, suggesting that interference by nonneutralizing antibodies, at least for the region encompassing residues 434 to 446, does not provide a mechanism for HCV persistence in chronically infected individuals.

Hepatitis C virus (HCV) has infected approximately 180 million people worldwide (2). Following infection, most people fail to clear the virus, and a chronic infection, often with serious sequelae, ensues (1, 38). HCV-related end-stage liver disease is the leading indication for liver transplantation, and reinfec tion of the grafted liver occurs rapidly (32). A systematic review of the research literature recently suggested that there is little, if any, benefit gained by the treatment of liver transplant recipients with standard antiviral regimens (24), and possible adverse effects associated with newly emerging direct-acting antivirals may limit their usefulness in this clinical setting. Antibodies are usually well tolerated, and the successful administration of anti-hepatitis B virus immunoglobulin (1g) (HBIG) (50, 59) sets an important precedent for HCV. The administration of HCV-neutralizing antibodies during the anhepatic phase and following transplantation could likewise prevent the reinfec tion of the grafted liver; the reduced incidence of HCV in individuals receiving HBIG containing anti-HCV antibodies (20) supports this notion. However, to date, the therapeutic administration of serum immunoglobulin or monoclonal antibodies targeting HCV has been disappointing (10, 51), indicating that further studies of the polyclonal response are needed, if we are to harness the opportunity that antibody therapy offers.

There is also an urgent need for the development of safe and effective HCV vaccines to prevent infection. Significant progress has been made toward T-cell-based vaccines (22), but these vaccines will not be sufficient to elicit sterilizing immunity. Consequently, the development of an antibody-targeted vaccine is still a priority. Protective vaccines will have to overcome significant viral antigenic diversity. HCV can be classified into seven genetically distinct genotypes and can be further subdivided into at least 70 subtypes, which differ by approximately 30% and 15% at the nucleotide level (29, 53). Within an infected individual, the virus exists as a quasispecies composed of genetically related yet distinct variants, and this variability allows the virus to escape host immunity (52). The envelope glycoproteins E1 and E2 are the natural targets of the neutralizing antibody response and are two of the most variable HCV proteins (8). E1 and E2 are N-linked glycosylated trans-membrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic membrane anchor (reviewed in reference 25) that mediate interactions with a range of cell surface molecules that result in entry via endocytosis. HCV entry requires interactions with a number of cell receptors, which include CD81, scavenger receptor class B type I (SR-BI), and members of the claudin and occludin tight-junction family of proteins (3, 5, 6, 16, 48a, 64, 67).

The development of a potent early neutralizing antibody response is associated with a spontaneous resolution of acute infection (12, 48). Antibodies that target both restricted and broadly neutralizing epitopes have been described. In general, antibodies with a restricted range target the first hypervariable region of E2, and quasispecies evolution leads to rapid escape (68). A number of more broadly neutralizing antibodies that target linear and conformational epitopes overlapping discontinuous regions of E2 involved in CD81 binding have been described (28, 31, 34, 44, 47,
One of these regions, encompassing amino acids (aa) 412 to 423 (in reference to the sequence of the H77c molecular clone), is targeted by the broadly neutralizing monoclonal antibodies (MAbs) AP33 and 3/11 (56). Antibodies that target this region are rare in natural infection, suggesting that this region is poorly immunogenic (55).

A number of mechanisms have been proposed to explain how HCV can persist in the presence of neutralizing antibodies. These proposed mechanisms include genetic escape (61), the occlusion of neutralizing epitopes through glycan shielding (18, 26) and lipid associations (23, 54), infection enhancement via serum components such as high-density lipoprotein (HDL) and apolipoproteins (13, 14, 60), and cell-to-cell transmission (7, 63). An additional mechanism was recently proposed by Zhang and colleagues, who reported that a linear region of E2 encompassing amino acids 434 to 446 (the so-called epitope II) elicits nonneutralizing antibodies in humans and chimpanzees that can inhibit the neutralizing activity of antibodies targeting aa 412 to 423 (65, 66). However, previous reports suggested that murine monoclonal antibodies, for example, MAbs 2/69a, 7/16b, and 11/20c, whose epitopes overlap the region encompassing aa 434 to 446, neutralize autologous H77c pseudovirus (HCVpp) infection and CD81 binding (9, 27). If both findings are true, then this suggests that antibodies that target the region encompassing aa 434 to 446 differ in phenotype according to their epitope specificity or that antibodies that target this region exhibit a dual phenotype.

To better understand the interplay between these groups of antibodies, we isolated human Ig fractions by peptide immunofinity techniques and characterized the resulting antibodies together with defined murine MAbs. The neutralizing phenotype, degree of cross-competition, and epitope specificity were assessed. Contrary to previous reports, we failed to observe inhibition or competition between antibodies specific for these two groups. Our data show that human antibodies that target the region encompassing aa 434 to 446 neutralize HCVpp and HCVcc infection. Importantly, these antibodies augment neutralization mediated by antibodies that target the region encompassing aa 412 to 423.

**MATERIALS AND METHODS**

**Peptides and MAbs.** Biotinylated peptides were synthesized by using Synphage PA lanterns (Mimotopes, Melbourne, Australia), using a C-terminal biotinyl residue separated from the epitope sequence by a Gly-Ser-Gly spacer sequence. Peptide purity was assessed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometry. Peptides were initially dissolved in dimethyl sulfoxide (DMSO) and then dissolved in phosphate-buffered saline (PBS) to a final concentration of 1 mg/ml in PBS. The peptides corresponded to amino acids 412 to 423 (QLINTN3SWGHN [peptide I]) and amino acids 434 to 446 (NTGWLAGFYYQHK [peptide II]) of the HCV H77c polyprotein and a control peptide corresponded to a seroreactive region of the rabies virus glycoprotein (VNLHDFRSD)IE) (11)). Where appropriate, anti-E2 MAbs, for example, MAbs 2/69a, 7/16b, and 11/20c, that target peptide corresponded to a seroreactive region of the rabies virus glycoprotein. Where appropriate, anti-E2 MAbs, for example, MAbs 2/69a, 7/16b, and 11/20c, that target peptide corresponded to a seroreactive region of the rabies virus glycoprotein.

**Patient samples.** Patient samples were obtained with ethics committee approval from the Trent HCV Cohort Study. Sera from 557 HCV antibody-positive (third-generation enzyme-linked immunosorbent assay [ELISA]; Ortho Diagnostics) individuals chronically infected with diverse genotypes of HCV encompassing residues 409 to 450 was performed by using ClustalX. Peptide sequences used in this study are highlighted by underlined sequences in strain H77c.

**Peptide-based immunoaffinity purification.** Streptavidin-coated M280 Dynabeads (Invitrogen) were coated with biotin-labeled peptides, representing the amino acid sequences of strain H77c encompassing amino acids 412 to 423 or 434 to 446, according to the manufacturer’s instructions. Beads were incubated with 400 μl of patient sera for 4 h at room temperature, washed six times, and incubated with antibody elution buffer (Thermo) for 10 min. Following the removal of the beads, antibody-containing solution was neutralized with 1 M Tris (pH 8.5). As a negative control, serum was incubated with streptavidin-coated Dynabeads in the absence of peptide. Antibody-containing solutions were aliquoted and stored at −20°C for further assays.

**Epitope mapping by enrichment of random peptide phage display libraries.** Purified Ig fractions were used to selectively enrich a 12-mer random peptide phage display library (NEB), as described previously (56). Following three rounds of enrichment, individual phage clones were isolated, and the peptide insert was determined by using a 96 sequencing primer (NEB). Sequencing products were resolved by using an ABI 3100 capillary sequencer. The deduced peptide insert amino acid sequences and the sequence corresponding to amino acids 412 to 423 or 434 to 446 of the H77c strain were aligned by using ClustalX (58). Enzyme immunoassays (EIAs) were also performed to determine the reactivities of the Ig fractions and control MAbs to the enriched phage clones. Approx-
cells infected with HCVpp were lysed with 20 mM nonessential amino acids. Plasmids containing JFH-1, H77c/JFH-1, (DMEM) (Invitrogen) supplemented with 10% fetal calf serum and 0.1% "fast" detergent were used to produce HCVcc as described previously (55), a total of 557 distinct sera obtained from individuals infected with diverse genotypes of HCV were screened for the presence of antibodies directed to a peptide representing amino acids 412 to 423 of strain H77c (peptide I). Extending our previously reported analyses (55), a total of 557 distinct sera obtained from individuals infected with diverse genotypes of HCV were screened for the presence of antibodies directed to a peptide representing amino acids 412 to 423 of strain H77c (peptide I).

RESULTS

Individuals with antibodies that target the region of E2 encompassing aa 412 to 423 frequently harbor antibodies that recognize the region of E2 encompassing aa 434 to 446. Extending our previously reported analyses (55), a total of 557 distinct sera obtained from individuals infected with diverse genotypes of HCV were screened for the presence of antibodies directed to a peptide representing amino acids 412 to 423 of strain H77c (peptide I).
Seventeen (2.3%) sera possessed peptide I-reactive antibodies. To investigate the presence of antibodies directed to the peptide representing amino acid residues 434 to 446 (peptide II), peptide binding assays were performed with those sera that were reactive with peptide I. Twelve of the 17 sera (70%) also reacted with peptide II, suggesting that these two epitopes are often coimmunogenic despite being recognized by the sera of only a small subset of patients. We then analyzed a subset of the peptide I-negative sera for binding to peptide II, which showed that the prevalence of reactivity to the region encompassing residues 434 to 446 was much higher than the prevalence of antibodies to the region encompassing residues 412 to 423. Despite the fact that the target peptides were based upon a genotype 1a strain (H77c), peptide-reactive antibodies were detected in sera from individuals infected with a range of genotypes. This suggested some degree of epitope conservation despite the observed sequence heterogeneity across aa 434 to 446 (Fig. 1).

To identify an appropriate high-titer serum sample for subsequent studies, and to define the longitudinal peptide I and peptide II binding profiles, sequential sera were tested for their abilities to bind to immobilized peptide I and peptide II (Fig. 2). While some differences in the relative binding were observed for each serum and peptide combination, the antibody response to both peptides in sera from both patients was maintained over a period of several years. The earliest available serum sample from patient 3A64 possessed high levels of peptide II-reactive antibodies but much lower levels of antibody reactivity to peptide I, while the other sera from the same patient had comparable reactivities to both peptides. One sample from each patient that exhibited detectable reactivity to both peptides was chosen for further study.

Affinity-enriched antibodies directed to peptide I and peptide II display specific binding to their respective peptides. Peptides corresponding to each E2 region were immobilized onto streptavidin-coated magnetic beads and used to affinity purify antibodies from sera from patients 1A76 and 3A64. This approach recovered between 10,000 and 40,000 ng of antibody per milliliter of serum. The resulting antibody fractions, as well as MAb AP33 and MAb 2/69a, demonstrated a level of dose-dependent binding to the target peptide that was at least 4-fold higher than that of binding to the reciprocal peptide (Fig. 3).

Peptide I-Ig and peptide II-Ig do not cross-compete for E2 binding. Having purified peptide I- and peptide II-specific immunoglobulin fractions (here termed peptide I-Ig and peptide II-Ig), the accessibilities of their epitopes on H77c E1E2 were assessed (Fig. 4). MAb AP33 and MAb 2/69a recognized Galanthus nivalis agglutinin (GNA) captured E1E2, although the latter MAb bound with a lower relative affinity. Similarly, affinity-purified Ig fractions also bound H77c E1E2 but with differing affinities. Peptide I-Ig derived from sera of patient 3A64 (3A64 peptide I-Ig) showed greater binding to E1E2 than did peptide II-Ig. However, the opposite was true for the Ig derived from sera of patient 1A76 (Fig.
Human and murine antibodies that target regions of E2 encompassing aa 412 to 423 and aa 434 to 446 neutralize HCVpp entry but differ in their neutralization breadths. Dilutions of 1A76 peptide I-Ig (●), 3A64 peptide I-Ig (○), 1A76 peptide II-Ig (□), 3A64 peptide II-Ig (○), MAb AP33 (▲), or MAb 2/69a (△) were used to neutralize the infectivity of H77c HCVpp (A) or JFH-1 HCVpp (B). Two different negative controls were used for the neutralization assays (◇): a normal human serum sample mock purified using the magnetic bead process was used as a negative control in the Ig neutralization assays, whereas HIV-1-specific monoclonal antibody 2F5 was used as a negative control in the MAb neutralization assays. Although neutralization assays were performed at the same time, for clarity, they are plotted on two panels corresponding to each peptide Ig and a third panel for the monoclonal antibodies. The same negative-control NHS data set is included in both Ig panels. The mean infectivities observed at the highest concentrations of each test antibody and the negative-control antibody were compared by using a t test. P values are indicated as follows: ns, P > 0.05; *, P < 0.01; ***, P < 0.001; ****, P < 0.0001. The mean luminescences observed for the uninhibited H77c HCVpp and JFH-1 HCVpp were 3,830 and 3,819 relative light units (RLU), respectively.

Peptide I-Ig and peptide II-Ig neutralize H77c HCVpp infectivity but differ in their abilities to neutralize JFH-1 HCVpp. We next determined the abilities of the peptide I-Ig and peptide II-Ig fractions and MAbs AP33 and 2/69a to neutralize HCVpp-expressing strain H77c (genotype 1a) and JFH1 (genotype 2a) E1E2 glycoproteins. A dose-dependent neutralization of H77 HCVpp was observed for all of the Ig fractions and MAbs tested (Fig. 5A). Differences in neutralization potency were evident, with estimated 50% inhibitory concentrations (IC50s) ranging from 0.1 to >8 μg ml⁻¹ for the different peptide I-Ig and peptide II-Ig fractions and monoclonal antibodies. MAb AP33 was the most potently neutralizing, while MAb 2/69a was the least neutralizing. When tested with HCVpp supplemented with E1E2 of the JFH-1 strain, a much lower neutralization potency was observed at the concentrations tested (Fig. 5B). A dose-dependent inhibition of entry was achieved by using the 1A76 peptide I-Ig and the 3A64 peptide II-Ig preparations as well as MAb AP33. In contrast, 1A76 peptide II-Ig, 3A64 peptide I-Ig, and MAb 2/69a did not neutralize JFH-1 HCVpp. This finding suggests that although the antibodies isolated from patients recognize the same peptide sequence, they recognize different epitopes, resulting in different neutralizing phenotypes.

Peptide I-Ig and peptide II-Ig potently neutralize H77c chimeric HCVcc infectivity but differ in their abilities to neutralize wild-type JFH-1 HCVcc. To assess whether the Ig fractions were capable of neutralizing the entry of authentic HCVcc virions, neutralization assays were performed by using cell culture-grown JFH-1 virus and a JFH-1 chimera possessing the structural proteins derived from clone H77c (Fig. 6). H77/JFH-1 was neutralized by peptide I-Ig and peptide II-Ig isolated from patients 1A76 and 3A64 as well as by MAb AP33 and MAb 2/69a. Again, the neutralization of wild-type JFH-1 HCVcc was more variable (Fig. 6B). Dose-dependent neutralization, which differed in potency, was observed for 1A76 peptide I-Ig, 3A64 peptide I-Ig, 3A64 peptide II-Ig, and MAb AP33. In contrast, both 1A76 peptide II-Ig and MAb 2/69a failed to neutralize JFH-1 HCVcc at the antibody concentrations tested.

Antibodies that target regions of E2 encompassing aa 412 to 423 and aa 434 to 446 show additive neutralization. Having shown
that antibodies that target the region of E2 encompassing aa 412 to 423 and aa 434 to 446 bind E2 independently of antibodies that target the region encompassing aa 412 to 423 and that both groups have a neutralizing phenotype, we assessed whether or not antibodies to these distinct regions could mediate additive neutralization. To assess this, increasing doses of peptide II-Ig were mixed with autologous peptide I-Ig at or near its estimated IC50 and then used to neutralize H77c HCVpp (Fig. 7A) or JFH-1/H77c HCVcc (Fig. 7B). Dose-dependent additive neutralization was observed for antibodies derived from both patients 1A76 and 3A64, with maximal combined neutralization reaching 93%. Combinations of MAbs AP33 and 2/69a resulted in the same additive neutralization, demonstrating that both human and murine antibodies share this property.

Peptide mapping of epitopes recognized by peptide I-Ig, peptide II-Ig, and MAb 2/69a reveals the presence of multiple epitopes. We have previously enriched random peptide display libraries to map the residues critical for MAb AP33 (56). Therefore, we adopted the same approach to determine residues involved in the binding of peptide I-Ig, peptide II-Ig, and MAB 2/69a. Following 3 to 4 rounds of biopanning, the binding of individual phage clones to the target Ig/MAb was assessed, and their peptide inserts were determined by DNA sequencing. Six of the seven phage clones isolated using 3A64 peptide I-Ig were strongly reactive to the selecting peptide I-Ig. Clone 6 had a lower level of reactivity, although this level of reactivity was still more than twice that observed for the negative control normal human serum (NHS) IgG (Fig. 8A). An alignment of the deduced amino acid sequences of the peptide inserts identified key residues that appeared to be critical for binding (Fig. 8A). One peptide sequence was identified in 7 independent clones, and residues that aligned to those present in the corresponding H77c sequence were L413, I414, N415, T416, G418, S419, W420, and I422. Analysis of all of the peptide sequences showed that the most frequently observed amino acid residues aligned to the sequence 416TxGxW420.

A similar analysis using 1A76 peptide I-Ig showed that three of four clones isolated were well recognized by the target Ig fraction in the phage binding immunoassay (Fig. 8B). The most prevalent phage clone sequence was present in 50% of the clones selected. This sequence possessed residues that mapped...
to positions L413, N415, and W420. The remaining clones were typified by the presence of a tryptophan, corresponding to residue W420, and either L413 or I414. The clone that was non-reactive in the phage immunoassay (clone 18) contained a tryptophan but did not contain any other amino acids that aligned to the E2 sequence.

Similar analyses of the antibodies that target the E2 region corresponding to peptide II were also able to identify key residues involved in binding. Seven clones enriched by 3A64 peptide II-Ig were selected and analyzed, and of these clones, two showed specific reactivity to the selecting antibody in the phage immunoassay (Fig. 8A). Sequence analysis of these clones revealed that the most frequent canonical binding sequence was GWLxG, corresponding to E2 residues G436, W437, L438, and G440. All phage-displayed peptides enriched by peptide II-Ig isolated from sera of patient 1A76 also demonstrated specific binding in the phage enzyme immunoassay, and analyses of the peptide sequence revealed that these antibodies recognized an overlapping yet distinct epitope consisting of G436, W437, and L441. Finally, the residues involved in recognition by MAb 2/69a were defined (Fig. 8C). Key residues were identified as G440, Y443, and K446. Together, the peptide mapping data demonstrate that antibodies directed to each of these peptides recognize overlapping yet distinct epitopes.

**DISCUSSION**

Studies of both sera and human monoclonal antibodies have shown that cross-neutralizing antibodies arise during chronic HCV infection (4, 19, 28, 31, 34, 37, 39, 41, 44, 45, 57). Several mechanisms have been proposed to explain how HCV persists. One mechanism proposed recently was the induction of nonneutralizing antibodies that target epitopes within the E2 region encompassing amino acid residues 434 to 446, which reportedly interfere with antibodies that target part of the E2-CD81 binding site. In particular, these antibodies targeted broadly neutralizing epitopes located in a highly conserved region of E2 corresponding to amino acids 412 to 423 (65, 66). This finding was contrary to previous reports that murine monoclonal antibodies to the region encompassing aa 434 to 446 neutralized E2-CD81 binding (9) and autologous H77c HCVpp infectivity (27). In order to clarify this, we studied the prevalences and neutralizing phenotypes of antibodies that target these two regions from sera of two chronically infected individuals. Crucially, our data, derived by using affinity-purified human Ig fractions, showed that human antibodies that recognize both of these regions neutralized HCVpp and HCVcc. We did not observe any interference between these two groups of antibodies. The depletion of antibodies that target the region encompassing aa 412 to 423 and aa 434 to 446 with satu-
rating amounts of peptide had a minimal effect on the overall neutralizing titer (data not shown), confirming our previously reported observation that antibody specificities to these epitopes contribute only a small part of the total polyclonal neutralizing response in chronically infected subjects (55).

Analysis of sera obtained from more than 500 individuals infected with a broad range of HCV genotypes revealed that the overall prevalence of peptide I-targeting antibodies was less than 3%, in accordance with our previously reported estimate (55). In contrast, Zhang et al. reported previously that two of nine chron-

FIG 8 Epitope mapping by enrichment of random peptide phage display libraries reveals the presence of distinct yet overlapping epitopes within regions of E2 encompassing aa 412 to 423 and aa 434 to 446. Affinity-purified Ig from patients 3A64 (A) and 1A76 (B) and Mab 2/69a (C) were used to enrich phages from a 12-mer random peptide library. Following three to four rounds of biopanning, individual phage clones were isolated, amplified, and tested for reactivity to the selecting antibody, and the amino acid sequence of the peptide insert was determined by DNA sequencing. The resulting sequences were aligned to the corresponding region of the H77c E2 amino acid sequence. The phage clone identification is presented, and individual residues aligning to the H77c sequence are shaded. Numbers in parentheses indicate the number of times each peptide sequence was recovered. The reactivity of each phage to the selecting antibody in a phage capture enzyme immunoassay is shown alongside each peptide sequence and is shown as the relative optical density compared to that of capture by an antibody preparation from an HCV-negative donor (NHS-Ig).

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ically infected patients harbored antibodies specific for a slightly extended (aa 412 to 425) peptide I (66). It is possible that extending the peptide increased the detection of antibodies with different specificities; however, this is not supported by previously reported mapping experiments, which suggested that the additional residues had no impact on antibody recognition (65, 66). Similar to the findings reported previously by Zhang et al., we confirmed that antibodies that target the region encompassing aa 412 to 423 coexisted with those that target the region encompassing aa 434 to 446.

Random peptide phage display epitope mapping experiments showed that both regions contained overlapping, yet distinct, epitopes. Phage binding enzyme immunoassays enabled the identification of specific binding mimotopes, thereby increasing our confidence that the canonical sequences identified key residues involved in binding. This is important, as the biopanning process can also enrich for irrelevant peptides (40). Some of our selected phage-displayed peptides did not demonstrate specific binding to their selecting antibody despite possessing sequence motifs that would implicate specific enrichment during selection. It is possible that the affinity of the interaction between these phage-displayed peptides and the target antibody falls below the sensitivity of our assays. Further experiments are under way to investigate this possibility.

Analysis of the peptide sequences of phages enriched using peptide I-Ig from both patients 1A76 and 3A64 revealed a consistent preservation of tryptophan, corresponding to W420, which is encompassed within the proposed 2/69a epitope. Together, these findings would argue against there being both neutralizing and interfering antibodies against this region. Random peptide phage display epitope mapping experiments showed that both regions contained overlapping, yet distinct, epitopes. Phage binding enzyme immunoassays enabled the identification of specific binding mimotopes, thereby increasing our confidence that the canonical sequences identified key residues involved in binding. This is important, as the biopanning process can also enrich for irrelevant peptides (40). Some of our selected phage-displayed peptides did not demonstrate specific binding to their selecting antibody despite possessing sequence motifs that would implicate specific enrichment during selection. It is possible that the affinity of the interaction between these phage-displayed peptides and the target antibody falls below the sensitivity of our assays. Further experiments are under way to investigate this possibility.

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