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Nonneutralizing Human Antibody Fragments against Hepatitis C Virus E2 Glycoprotein Modulate Neutralization of Binding Activity of Human Recombinant Fabs

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Evidence from clinical and experimental studies indicates that hepatitis C virus E2 (HCV/E2) glycoprotein is the major target of a putatively protective immune response. However, even in the presence of a vigorous production of anti-HCV/E2 antibodies, reinfection can occur. Dissection of the human immune response against HCV/E2 indicated that blocking of binding of HCV/E2 to target cells [neutralization of binding (NOB) activity] varies widely among antibody clones. Moreover, *in vivo,* simultaneous binding of antibodies to distinct epitopes can induce conformational changes and synergies that may be relevant to understanding the anti-HCV immune response. In this study, human recombinant Fabs were generated by affinity-selecting a phage display repertoire library with antibody-coated HCV/E2. These Fabs, which share the same complementarity-determining region DNA sequences, had higher affinity than other anti-HCV/E2 Fabs but showed no NOB activity even at the highest concentrations. Binding of Fabs to HCV/E2 caused conformational changes modifying Fab-binding patterns and reducing, with a negative synergistic effect, Fab-mediated NOB activity. These data suggest that some antibody clones have the potential to modify HCV/E2 conformation and that, in this state, binding of this glycoprotein to its cellular target is less prone to inhibition by some antibody clones. This can explain why high anti-HCV/E2 antibody titers do not directly correlate with protection from infection. Information on the interactions among different antibody clones can contribute to understanding virus–host interplay and developing more effective vaccines.

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of blood-borne non-A non-B hepatitis (Choo *et al.,* 1989; Kuo *et al.,* 1989). After primary HCV infection, virus persistence occurs in a large proportion of cases (70–80%) and involves significant risk of progression to liver cirrhosis and hepatocellular carcinoma (Alter *et al.,* 1992). Therapy is only partially effective, with only 20 to 30% long-term response in patients treated with the current therapy centered on interferon-α (Fried and Hoofnagle, 1995). In this context, the development of new therapeutic compounds and the design of an effective vaccine have become priorities for the scientific community.

Currently, the first issue in designing a vaccine is the precise identification of the viral and host components involved in the elicitation of protective immunity. There is now consensus on the existence of HCV-neutralizing antibodies based on clinical and experimental data. In humans, immunoglobulins have proven effective in the prophylaxis of HCV infection (Piazza *et al.,* 1997). Moreover, protection of chimpanzees has been achieved following immunization with glycoproteins E1 and E2; this phenomenon has been linked to the induction of specific anti-E2 antibodies (Choo *et al.,* 1994), commonly referred to as antibodies with neutralization of binding (NOB) activity, which are able to neutralize the binding of E2 to susceptible cells. Although the assessment of the capacity of NOB antibodies to inhibit HCV infection has been hampered by the fact that HCV does not grow efficiently in cell cultures, high titers of NOB antibodies have been associated with the natural resolution of HCV infection (Ishii *et al.,* 1998). Other studies have failed to demonstrate a protective effect of these antibodies (Farci *et al.,* 1992; Lai *et al.,* 1994). This contrasting evidence may depend on the fact that the antibody response against glycoprotein E2, a key viral protein that interacts with the putative cellular receptor CD81 (Pileri *et al.,* 1998), seems to be highly heterogeneous, at least when assayed by NOB activity. Moreover, recent reports describing the dynamics of intrahost evolution of HCV populations during primary infection have shown that a crucial phase for disease outcome (i.e., resolution vs persistence) lies at a time point corresponding to the production of antibodies by the infected host (Farci *et al.,* 2000; Manzin *et al.,* 2000). Altogether, these data strongly suggest that the role of antibodies in HCV infection, though requiring

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further study (Cerny and Chisari, 1999), is crucial as may well be expected of a disease caused by a viral agent able to finely cope with the immune system for its survival in the host.

Recently, our group dissected the antibody response of an HCV-positive patient by generating a large panel of human monoclonal antibody fragments specific for E2, demonstrating a remarkable heterogeneity of the clones, at least when assayed by determination of NOB activity (Burioni *et al.,* 1998b). However, an important role *in vivo* can be played by nonimmunodominant antibodies or by the simultaneous binding of antibodies to distinct epitopes, which induces synergies or conformational changes. For these reasons, a phage display combinatorial library containing the antibody repertoire of an HCV-infected patient was affinity-selected using antibody-coated E2 glycoprotein. As a result, genes coding for a novel family of human recombinant Fabs directed against HCV/E2 glycoprotein were cloned and used for production of human recombinant Fabs in bacteria. These Fabs, which failed to be selected with procedures using non-antibody-coated antigens despite exhaustive screening, were characterized with regard to their affinity and for their ability to induce conformational antigen changes. Moreover, their synergistic effect on the NOB activity of other human recombinant anti-HCV/E2 Fabs was investigated. The data shown here, revealing in detail the nature of the humoral immune response against a key HCV antigen, may improve our understanding of the virus–host interplay and may contribute to the design of vaccines eliciting more effective responses.

RESULTS

Affinity selection of the phage library and anti-HCV Fab generation

Affinity selection of the phage display combinatorial library using solid-phase-bound HCV/E2 glycoprotein coated with purified recombinant anti-E2 human Fabs yielded a 15-fold enrichment of the eluted phage over four rounds of panning. Genes coding for putative anti-HCV/E2 were inserted in a soluble Fab expression vector and transformed bacteria were used as a source of recombinant human Fabs. When analyzed in an ELISA test using the same antigen used for selection, 12 of 20 clones were demonstrated to react strongly with HCV/E2. Sequence analysis of the heavy chain variable part indicated that all the positive clones shared very similar sequences (maximum divergence of 2 nucleotides), allowing the grouping of all Fabs into a single family (Burioni *et al.,* 1998b). All complementarity-determining regions (CDR 1, 2, and 3) were identical in all Fabs. One of these Fabs, designated HCV/E2 e10B, was chosen, fully sequenced, purified by immunoaffinity, and further characterized.

The predicted amino acid sequence of the variable part of the heavy and light chains of Fab e10B, though corresponding to one expected from a human antibody variable part, diverged from previously described human antibodies, confirming its novelty; in particular, the sequences were demonstrated to differ from the other Fabs against HCV/E2 cloned from the same patient (Fig. 1). Analysis of the sequence over the IMGT database (Lefranc *et al.,* 1999) demonstrated that the heavy chain was derived from a V_H4 germline and the light chain from a V_K1 germline. Both genes showed a mutational pattern indicating an antigen-driven affinity maturation, as shown by the lower mutation rate in the framework region compared with the complementarity-determining regions (9.5% vs 41.1% in the heavy chain; 6.2% vs 25.5% in the light chain).

Affinity and competition binding

Relative affinity determinations showed that this Fab was endowed with a higher affinity than the other Fabs directed against the same antigen cloned from the same patient (Fig. 2) and present in the phage library utilized for affinity selection. Competition binding experiments demonstrated that, as expected, the binding of Fab e10B to HCV/E2 is not inhibited by any other Fab (Fig. 3A). In contrast, previous binding of Fab e10B inhibited the binding of Fab e8 to HCV/E2 (Fig. 3B). This effect was asymmetric, as e10B inhibited e8 but was not itself inhibited.

Reciprocal interactions between Fabs were also evaluated with soluble HCV/E2 in a sandwich ELISA with the competing Fab bound to solid support. HCV/E2 bound on any of the Fabs was accessible by FLAG-labeled e10B, as expected (data not shown). Binding of HCV/E2 to bound Fab e10B inhibited subsequent e8 and e10B binding, while having no effect on binding of e20, e137, e301, and e509 (Fig. 4).

Neutralization of binding activity and epitope mapping

Measurement of the NOB activity of the e10B clone showed that, despite its very high affinity, this Fab does not neutralize the binding of E2 to its cellular target even at the highest concentration used in our assay (40 μg/ml). In addition, the binding of this Fab to HCV/E2 decreased by 10 times the NOB activity of the most potent anti-HCV/E2 Fab available (e509), as shown in Fig. 5. Finally, epitope mapping of the region recognized by Fab e10B was carried out by pepscan analysis (Burioni *et al.,* 1998b). The mapping was unsuccessful (data not shown), suggesting that this antibody fragment recognizes a conformational epitope not reproduced by this approach.

DISCUSSION

The use of combinatorial libraries displayed on the surface of filamentous phage containing the immune

LIGHT CHAIN SEQUENCES

Fab#	fr1	cdr1	fr2	cdr2	fr3	cdr3	fr4
10B	MAELTQSPSFLSASVGDRVTITC	RASQGVTILLA	WYQQKPGKPPKALIY	AASSLQS	GVPSRFSGSGSDTDFTLTISSLQPEDSATYYC	QQLNTYPWT	FGQGT
8	MAELTQSPGTLSLSPGERATLSC	RASHRVNNNFLA	WYQQKPGQAPRLLIS	GASTRAT	GIPDRFSGSGSGTDFTLTISRLEPDDFAVYYC	QQYGDSPLYS	FGQGT
20	MAELTQFPSSVSASPGDRVTITC	RGSQGVSTYLA	WYQQKPGKAPQLLID	ATSNLQS	GVPSRFSGGGSGTDFTLTISSLQSEDFATYYC	QQYYNYPLT	FGGGT
137	MAELTQSPSFLSASVGDRVTITC	RASQGISNYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSWTEFTLTISRLQPEDFATYYC	QHLNTYPWT	FGQGT
301	MAELTQSPATLSVSPGERATLSC	RASQSVSSRLA	WYQQKRGQAPSLLIY	DTSSRAT	GVPARFSASGSGTQFTLTISSLQSEDFALYYC	QQYNDWPST	FGQGT
509	MAELTQVPATLSASPGERASLSC	RASQSVSSNLA	WYQQKPGQAPRLLIS	GASTRAT	GVPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWPPH	FGQGT

HEAVY CHAIN SEQUENCES

Fab#	fr1	cdr1	fr2	cdr2	fr3	cdr3	fr4
10B	LLESGPGLVKPSQTLSLTCTVSGV	SISYGGRGVSYWG	WVRQSPGKGLEWIGH	IYYFGDTFYNPSLNN	RATISIDSSKNQFSLKLKSVTASDTALYFCAR	STLQYFDWLLTREAAYSIDF	WGQGI
8	LLEQSGAEVKMPGATVKVSCQSSRY	TFTSYGIG	WVRQAPGQGLEWMGW	ISGYTHETKYAQSFQG	RVTMTAETSTGTAYMELRSLRSDDTATYYCAR	DGGGRWWWPPTHLRAFDV	WGQGT
20	LLEQSGAEVKKPGSSVKVSCKASGD	HYGIN	WVRQAPGQGLEWMGG	IIPVFGTTTYAQKFQG	RATITADDSTGTAFLELTRLTFDDTAVYFCAT	PHQLHVLRGGKALSPWDY	WGQGT
137	LLEQSGSEVKVPGSSLKVSCKTSGG	TFSTYTFS	WVRQAPGQGLEWMGG	ITPIIGIANYARNFQD	RVTITADESTSTVYMEVRRLRSEDTAVYYCAK	TSEVTATRGRTFFYSAMDV	WGQGT
301	LLEQSGSEVKKPGSSVRVSCTTSGG	TLSDYGFN	WLRQAPGQGPEWMGG	IIPLFRRTTYGQKFQG	RLTITADESTGATYMELSSLRSDDTAVYYCAR	EKVSVLTGGKSLHYFEY	WGKGT
509	LLEESGAEVKKPGSSVKVSCKTSGD	TFRYGIT	WVRQAPGQGLEWMGQ	IMPTFATATYAQRFQG	RVTISADESTSTAYLEVRSLRSEDTAVYYCAT	PRQVTILRGPKALSPWDY	WGQGT

FIG. 1. Deduced amino acid sequence of human anti-HCV/E2 Fab e10B variable heavy and light chains compared with anti-HCV/E2 human Fabs used for the epitope masking selection procedure, as previously described (Burioni *et al.*, 1998b). Fab DNA sequences are accessible through GenBank.

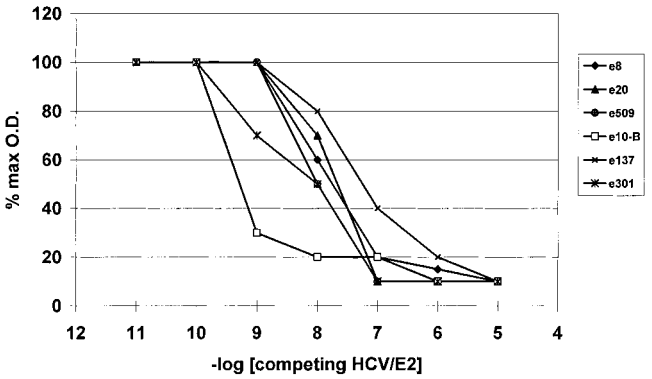

FIG. 2. Determination of relative affinity, estimated by inhibition ELISA. Data are expressed as a percentage of maximum OD_{450}.

repertoire of infected patients provides an efficient tool for dissecting accurately the host response to important viral antigens. However, certain epitope specificities may be missed because they are nonimmunodominant or because they become exposed only in particular situations, e.g., following conformational changes of the antigenic structure. *In vivo,* antigen exposure does not consist of the sole contact of the native viral antigen with the immune system, but also involves the simultaneous

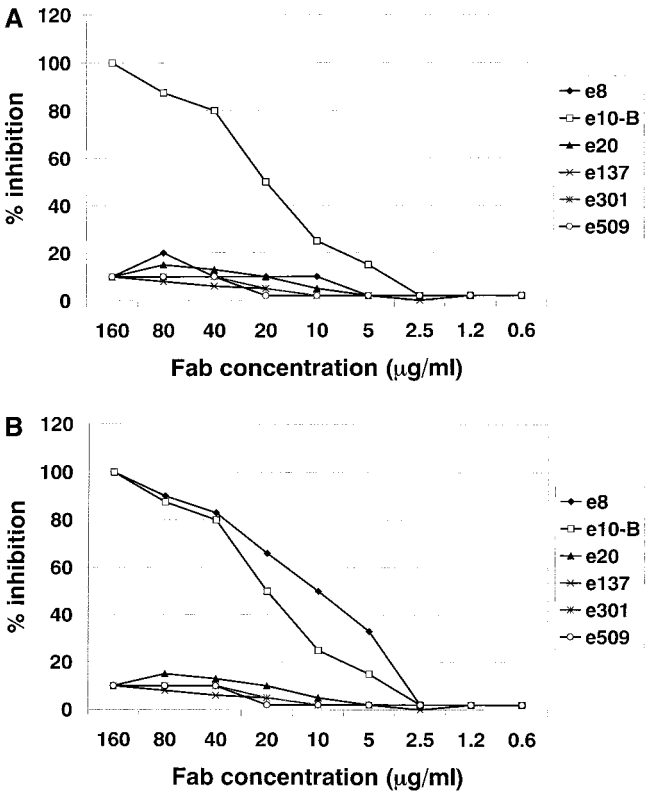

FIG. 3. (A) Inhibition of binding of FLAG–Fab e10B to the antigen by different concentrations of purified unlabeled Fabs. (B) Inhibition of binding of different FLAG–Fabs by previous binding of Fab e10B. Binding of the FLAG–Fabs was demonstrated by FLAG ELISA. Data are presented as a percentage of inhibition.

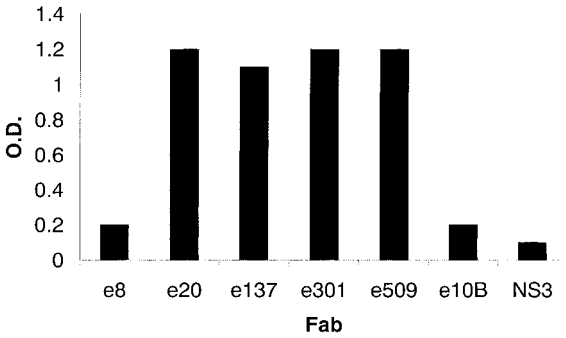

FIG. 4. Binding of different FLAG–Fabs to HCV/E2 bound to e10B. Binding of FLAG–Fab was demonstrated by FLAG ELISA.

binding of several antibodies to distinct epitopes, resulting in conformational changes and synergies among different clones representing discrete parts of the humoral immune response. Moreover, structural modifications induced by the binding of viral surface proteins to antibodies or receptors can be crucial for viral physiology and for the development of a protective host response.

This work indicates how the binding of human recombinant Fabs to HCV/E2 glycoprotein can unveil epitopes that would be inaccessible without this strategy despite the exhaustive screening of hundreds of different clones from the library representing the humoral repertoire of an HCV-infected patient. Previous coating of HCV/E2 with specific human Fabs allowed the molecular cloning of a novel family of human recombinant Fabs recognizing a discrete epitope and representing a part of the antibody response against HCV/E2 of this infected patient.

The epitope masking procedure may allow the cloning of human recombinant Fabs directed against weakly immunogenic epitopes (Ditzel *et al.,* 1995) that elicit antibodies with a lower affinity. This does not seem to be the case in this work, as measurement of the relative affinity of Fab e10B showed higher values than with other Fabs. The difficulties met when isolating phage bearing

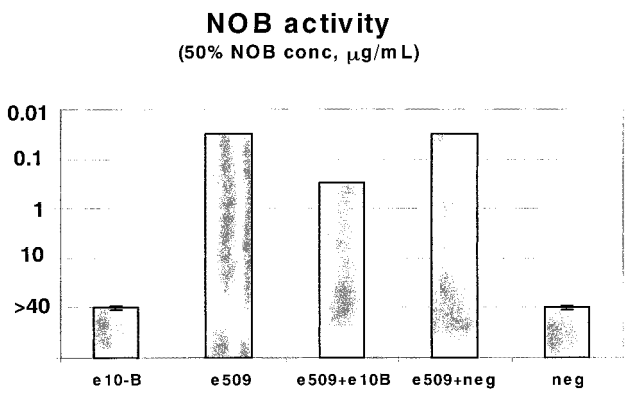

FIG. 5. Inhibition of binding of HCV/E2 to its cellular target by different Fabs. Fab C33-3 is an anti-HCV human Fab directed against NS3 protein (Plaisant *et al.,* 1997) used as a negative control.

this Fab without using antibody-coated HCV/E2 can be explained with a relatively low representation of this particular clone in the repertoire library. Less frequent clones may miss access to the target protein during the selection procedure even when endowed with equal or higher affinity; this phenomenon is usually due to saturation by more frequent clones (Ditzel *et al.,* 1995). Competition experiments showed that the presence of other anti-HCV/E2 Fabs did not affect the binding of Fab e10B, as expected considering the strategy by which the molecule was cloned. However, when Fab e10B was allowed to bind to HCV/E2 before the binding of the other Fabs was assayed, one of them (e8) could no longer bind to the antigen. Asymmetric inhibition (e10B inhibiting e8 binding and not vice versa) strongly supports the hypothesis that e10B is directed against a conformationally modified E2 that fails to be recognized by e8.

The study of the activity of Fab e10B in neutralizing the binding of E2 to cells by NOB measurement yielded negative results despite vigorous binding of the Fab to the antigen even at low concentrations and despite a relatively high affinity, suggesting that e10B recognizes a discrete region of E2 not directly involved in binding to the E2 cellular target. However, the NOB effect of the binding of Fab e10B is far from negligible, as its presence considerably reduced the activity of the Fab with the highest NOB effect described in our previous works (Burioni *et al.,* 1998b). Demonstration of lack of competition for binding to the antigen between Fab e509 and Fab e10B using soluble HCV/E2 ruled out the possibility that this effect might be due to competition between these two Fabs for binding to HCV/E2 in solution.

These data have an important role in the dissection of the anti-HCV/E2 response at the molecular level and in understanding the effects of the different components of the humoral immune response against this viral pathogen. Indeed, the presence of antibodies that, despite their high affinity, not only have no effect in neutralizing the binding of E2 to the cellular target, but considerably impair the NOB activity of other molecules, can explain the lack of NOB effect even in human sera with high titers against HCV/E2 (Rosa *et al.,* 1996). Furthermore, the identification of antibodies with these properties in the humoral repertoire of chronically infected patients can be crucial to understanding the reinfections that occur even in the presence of a vigorous immune response (Farci *et al.,* 1994; Lai *et al.,* 1994).

Although many attempts have been made to set up HCV *in vitro* neutralization tests (Shimizu *et al.,* 1994; Zibert *et al.,* 1995), this pathogen does not yet grow reproducibly and efficiently in cell cultures. At present, the only quantitative method available for measuring possible antiviral activity is NOB titer determination, even though its correlation with true neutralization activity remains to be proved, considering that for other viral pathogens inhibition of binding to cellular receptors does not correlate directly with neutralization (Barbas *et al.,* 1992a).

Finally, this work suggests that E2 is present in at least two conformational states, only one of which is recognized by Fab e8. It may be speculated that binding of e10B induces the conformational change. The availability of antibodies able to distinguish between the two hypothetical states of HCV/E2 can be crucial in shedding light on the interactions between viral and cellular structures, which could be at the basis of virus adsorption and entry.

In conclusion, the availability of a panel of Fabs representing the interactions of the human humoral immune system with HCV can be very useful for elucidating the efficacy of the immune response in protecting against this infection and for designing new strategies for immunization against HCV. Ideally, these vaccines should be able to elicit only neutralizing antibodies, avoiding the production of other nonprotecting immunoglobulins. Experience with other viral infections that, like HCV, become persistent despite the presence of a detectable antibody response (as is the case of HIV) suggests that protective antibodies are indeed generated, but that they are only a part of the immune response and are probably produced at a level insufficient to afford protection (Burton and Parren, 2000).

MATERIALS AND METHODS

Library construction and selection of antigen-binding phage through library panning

The construction of a phage display library containing the IgG1/κ antibody repertoire of a patient positive for HCV antibodies and HCV RNA in the serum (genotype 1b) (Okamoto *et al.,* 1992) has been described previously (Burioni *et al.,* 1998b). Panning of the library without epitope masking was conducted as described previously. In brief, four ELISA wells (Costar, Corning, NY) were coated overnight with 400 ng/well of HCV (genotype 1a) glycoprotein E2 (Lesniewski *et al.,* 1995). Subsequently, plates were washed four times with water and blocked with PBS/3% BSA for 1 h at 37°C. The blocking solution was discarded, and 50 μl of phage resuspended in PBS/1% BSA was added to each well and incubated for 2 h at 37°C. Unbound phage were removed by washing carefully 10 times with PBS containing 0.5% Tween 20. Bound phage, bearing on the surface antigen-binding Fabs, were eluted with acid and amplified, as described, by *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA) infection and with VCS-M13 helper phage (Stratagene) superinfection. After four cycles of panning, the pComb3 phage display vector was transformed into pComb3/CAF soluble Fab expression vector (Burioni *et al.,* 1998a) and transformed *E. coli* were used as a source of Fabs, which were produced, purified, and quantified as described (Barbas *et al.,* 1992b; Ditzel *et al.,* 1995). For production of FLAG-labeled Fab, genes were inserted in the pComb3/

FLAG vector (R. Burioni, submitted for publication), with an epitope added in the carboxy-terminal end of the heavy chain fragment recognized specifically by a mouse anti-FLAG monoclonal antibody.

Epitope masking panning procedure

Epitope masking panning was performed as described (Ditzel *et al.,* 1995) with some modifications. In brief, after the well was blocked with PBS/3% BSA, 70 μl of a mixture containing previously described (Burioni *et al.,* 1998b) purified human monoclonal recombinant anti-HCV/E2 Fabs e8, e20, e137, e301, and e509 (10 μg/ml each clone) was added to wells and incubated for 1 h at 37°C. After incubation, 50 μl of the mixture was removed and 50 μl of phage was added, incubated, washed, eluted, and amplified as above.

ELISA and competition ELISA analyses of recombinant Fabs

The reciprocal interactions among different Fabs were defined by inhibition ELISA. Briefly, antigen-coated plates were blocked and 50 μl of a purified preparation of competing Fabs at known concentration was added to the wells and incubated for 2 h at 37°C. After this step, an appropriate amount of probe FLAG-labeled purified recombinant Fab (FLAG–Fab) was added directly to wells to obtain a final concentration that gave approximately 70% of the maximum OD_{450} (optical density measured at 450 nm) in ELISA and was incubated for an additional 30 min. Plates were then washed 10 times with PBS/0.05% Tween and binding of the FLAG–Fab probe to the antigen was revealed with anti-FLAG M2 mouse monoclonal antibody (Sigma, St. Louis, MO; 10 μg/ml in PBS). Plates were washed as described above and binding of mouse monoclonal antibody was demonstrated by addition of peroxidase-conjugated anti-mouse antibodies (Sigma; 1:700 in PBS). After a final wash, 100 μl of substrate (Sigma) was added and plates were read for optical density at 450 nm after 30 min at room temperature in the dark. Final results were determined as a percentage of inhibition with the formula: percentage inhibition = 100 × (OD_{450} of probe FLAG–Fab alone − OD_{450} of probe FLAG–Fab with competitor Fab)/OD_{450} of probe FLAG–Fab alone.

For determination of the reciprocal interactions between Fabs and HCV/E2 in solution, ELISA plates were coated with the competing purified Fab at a concentration of 100 ng/well at 4°C overnight. After washing and blocking were performed as described above, 40 μl of HCV/E2 at a concentration of 50 μg/ml in PBS was added to each Fab-coated well and incubated for 2 h at 37°C. After this step, FLAG–Fabs were added at a concentration yielding 70% of the maximum OD in ELISA and determined as described above. Experiments are the average of three assays performed in duplicate.

Relative affinity determination

Determination of relative affinity of purified Fab recombinant fragments was performed as described (Burioni *et al.,* 1998b; Burton *et al.,* 1991; Rath *et al.,* 1988). Briefly, appropriate amounts of purified Fabs were tested in an ELISA test in competition with different concentrations of the same HCV/E2-1a antigen used to coat the plates. Fab and antigen were added to the plate and incubated for 3 h at 37°C. ELISA was then performed as described above.

Neutralization of binding assay

Purified preparations of human recombinant Fabs were tested for their ability to neutralize the binding of recombinant HCV/E2 glycoprotein to MOLT-4 cells and 50% NOB concentration was determined as described (Burioni *et al.,* 1998b; Rosa *et al.,* 1996). When two Fabs were tested together, a mixture of the two molecules (1:1) was prepared in advance and used for NOB determinations. NOB activity was calculated as the concentration (in micrograms per milliliter) achieving 50% of neutralization of binding. All assays were performed blind at least in duplicate and repeated twice.

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