High-affinity self-reactive human antibodies by design and selection: Targeting the integrin ligand binding site
(drug design/cell adhesion/phage display/combinatorial libraries/metastasis)

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ABSTRACT A strategy for the design and selection of human antibodies that bind receptors is described. We have demonstrated the validity of the approach by producing semisynthetic human antibodies that bind integrins αβ and αββ with high affinity (10^{-10}-10^{-11} M). The selected antibodies mimic the integrins' natural ligands as demonstrated by their ability to compete with these ligands and Arg-Gly-Asp (RGD)-containing peptides for binding to the integrins. Furthermore, the antibodies bind in a cation-dependent fashion and are functional in cell adhesion assays. Antibodies that are high-affinity inhibitors of cell adhesion receptors should be of use in assessing receptor function and dissecting mechanisms of adhesion. Semisynthetic human antibodies that target integrins are potential therapeutic agents for the treatment of a number of diseases including thrombosis and metastasis. Furthermore, antibodies that are optimized to bind by a single complementarity determining region may be important lead compounds for the design of small molecule pharmaceuticals.

The potential for use of human antibodies in therapy and prevention of disease is enormous. The applications can be divided into the need for reactivity to exogenous antigens such as viruses or self antigens such as cell surface receptors. Although the combinatorial antibody library approach (1) provides immediate access to high-affinity human antibodies directly from immune individuals (2), the ability to generate antibodies to self antigens where tolerance mechanisms play a role remains a problem. Natural autoantibodies are generally of low affinity and specificity and are often directed against antigens or epitopes that are inappropriate for therapeutically applications (3). The use of semisynthetic combinatorial libraries, where molecular diversity is synthetically generated and targeted to the complementarity determining regions (CDRs) (4-7), offers a solution to the problem of generating antibodies to self antigens. Since sequence diversity is generated in vitro, tolerance is not an issue. Herein we demonstrate that the design and selection of high-affinity antibodies can be guided in the case of anti-receptor antibodies with a limited knowledge of the ligand with which the receptor interacts.

We have chosen to target the integrin family of cell adhesion receptors with semisynthetic antibodies to demonstrate the validity of the guided approach. Integrins are cell surface αβ heterodimers that mediate numerous cell–matrix and cell–cell contacts. Consequently, integrins direct many normal tissue remodeling events including fertilization, development, and immunological and inflammatory responses (8-11). Integrins also have a major impact on the pathology and progression of several diseases. For example, osteoporosis is influenced by αβ, which directs osteoclast adhesion to bone (12, 13). Atherosclerosis is an example of integrin-mediated adhesion gone awry. Integrin αβ participates in monocyte adhesion to the aortic wall forming the earliest atherosclerotic lesion, the fatty streak (14). Integrin αββ further exacerbates the lesion by enabling platelet adhesion and thrombus formation at the existing atherosclerotic plaque. Many microbes gain access to host by using integrins as entry portals to the cell. Yersinia pseudotuberculosis, an endotoxin complexed with the cytoplasm by binding integrin αβ and echovirus, which can result in viral meningitis, uses integrin αβ in a similar manner (15, 16). It is evident that a means of blocking integrin function with high fidelity would provide a therapeutic measure to combat many of these pathological conditions. However, given the ubiquitous role of the integrin gene family in cell adhesion, the key to such an approach is to target integrins with high-affinity inhibitors that maintain reasonable receptor specificity.

MATERIALS AND METHODS

Reagents Strains and Vectors. Oligonucleotides were from Operon Technologies (Alameda, CA). Escherichia coli, phage, and the phagemid vector pComb3 are as described (17). The human integrins αβ, αββ, and αββ were purified as described (18).

Library Construction and Selection. PCR mutagenesis was performed to construct a library of heavy chain CDR3 (HCDR3) variants. PCR amplification was performed as described (4) but with hiv12, a human antibody Fab fragment, as the template (19) and the following two oligonucleotide primers: (i) CTC-CTC-CTC-CTC-GAC-GTC-CAT-ATA-ATA-GCA-MNN-MNN-MNN-ATCCG-AGC-MNN-MNN-MNN-GCA-CCC-CAC-TCT-CGC-ACA-ATA-ATA and (ii) GCA-ATT-AAC-CTC-CAC-TAA-AGG-G, where N is A, C, G, or T, and M is A or C (The GenBank accession number of hiv12 is L03147.) The PCR product was gel-purified, digested with XhoI and Aat II, gel-purified, and ligated with XhoI/Aat II digested vector of clone 1 (6). Subsequent steps were as described (17) to produce phage displaying antibody fragments on their surface.

The integrin αβ, in 50 mM Tris base/150 mM NaCl/1 mM MgCl2/1 mM MnCl2/1 mM CaCl2, pH 7, was immobilized on Costar 3690 microtiter plates for selection. Selection and subsequent purification of isolated clones was performed as described (4, 7, 17).

Characterization of Integrin Binding Antibodies. The ability of selected Fab’s to compete with the integrin ligands for integrin binding was determined with a purified ligand–receptor binding assay (20-22). Cell adhesion assays were performed as described (23).

Abbreviations: CDR, complementarity determining region; HCDR3, heavy chain CDR3.

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RESULTS AND DISCUSSION

Semisynthetic or synthetic antibody libraries are an alternative to immunization for the production of antibodies. This approach involves the semisynthesis of antibody genes that allows for the controlled introduction of diversity into the combining sites of antibodies. The display antibody fragments on the surface of filamentous phage allows for the affinity selection of clones from vast libraries. We have demonstrated that antibodies can be selected to bind small haptens (4, 7), metal ions (6), and metal oxides by introducing a random sequence into one or two CDRs of an antibody. An alternative to a totally random search of binding sites for the desired specificity is a directed search that incorporates information known to be relevant to a given binding problem.

For anti-receptor antibodies, information relevant to the binding of the ligand to the receptor may be utilized to construct specialized libraries directed to bind a given receptor or receptor family. The strategy is illustrated in Fig. 1. When the sequence of a ligand has been determined to be directly involved in binding the receptor, that sequence or a minimal portion thereof may be transplanted into CDRs of a synthetic antibody library. Any of the six CDRs could be chosen as the site of transplantation and optimization of the sequence. Antibodies selected from such a directed library to bind the receptor might be expected to interact with the ligand binding site.

The Arg-Gly-Asp (RGD) sequence is an integrin recognition motif found in many adhesive proteins (8–11). Recently, this motif has been selected from a disulfide-constrained random peptide library (24). A functional RGD motif occurs in four structures: the snake venom "disintegrins" kistrin (25), echistatin (26), tenasin (27), and the foot and mouth disease virus (28). Three of these proteins are known ligands for $\alpha_\beta$ and/or $\alpha_{IV}\beta_1$, the integrins we have selected for this investigation. In each case, the RGD sequence is found in an extended flexible loop at the apex of the turn. In tenasin it is found within an immunoglobulin-like domain. Both receptors bind RGD-containing peptides, albeit with substantially lower affinity than their macromolecular ligands. The simple RGD motif is insufficient for high-affinity binding. Binding is dependent on the correct conformational display of the motif as shown by perturbations in affinity of peptides as a function of flanking sequences and conformational constraint conferred by cyclization (29). Thus we reasoned that simple placement of the RGD motif within a CDR would be insufficient to confer binding with a useful affinity and specificity. A natural human antibody containing the RGD sequence in the C-terminal portion of HCDR3 did not bind $\alpha_\beta$, $\alpha_{IV}\beta_1$, or $\alpha_\beta$ (C.F.B. and T. Jones, unpublished data). High-affinity binding should require placement of the motif at the apex of an extended loop with flanking regions that conformationally optimize the display of the motif.

The HCDR3 was chosen for display of the motif as the loop length and sequence in this region is extremely variable. The human antibody Fab fragment hiv12, an anti-gp120 clone, was chosen as the presenting scaffold as it is produced well in E. coli and has an extended HCDR3, 18 residues (19). Residues at the N- and C-terminal portions of the HCDR3 were maintained to retain the stem of the extended hairpin loop (30, 31). The RGD sequence was placed at Kabat positions 100–103 ensuring its location near the apex of the turn.

To optimize the conformational display of the motif, three codons on each side of the RGD sequence were randomized by PCR-directed mutagenesis using an oligonucleotide that anneals over the HCDR3. Randomization of the six codons was achieved with an NNK doping strategy (where N represents an equal mixture of deoxynucleotides A, C, G, and T and K represents an equal mixture of G and T). Mutagenesis was limited to six codons as the resulting library could encode $\sim 10^6$ nucleotide sequences, which is near the upper limit of proteins that can be surveyed using the present phage display system. Direct cloning of the PCR product was facilitated by a naturally occurring Aat II restriction site in C-terminal end of the HCDR3. The library was constructed in the filamentous phage surface display vector pComb3 (17). The library of $3 \times 10^9$ clones was selected for binding integrin $\alpha_\beta$ by four rounds of panning against immobilized receptor. Subsequent screening of the soluble Fab in a simple ELISA revealed binding to $\alpha_\beta$. Sequencing of 10 clones revealed 5 were unique. Examination of the sequences revealed homologies within the group and with other known ligands. The SFG-RGD sequence of Fab-9 is found in lamB (32), IP-RGD is in kistrin (25), and RGD-W is in a number of other snake venoms (33). The most interesting feature of the selected sequences is the strict conservation of RGD-XXR, which is not

![Design and selection of human anti-receptor antibodies](image)

**Table 1.** Inhibition constants and amino acid sequences of RGD-containing antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$IC_{50}$, M</th>
<th>$\alpha_\beta$</th>
<th>$\alpha_{IV}\beta_1$</th>
<th>$\alpha_\beta$</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>$2.5 \times 10^{-10}$</td>
<td>$2.5 \times 10^{-10}$</td>
<td>$5 \times 10^{-10}$</td>
<td>TQQ-RGD-WRS</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>$2.0 \times 10^{-10}$</td>
<td>$5.0 \times 10^{-10}$</td>
<td>NI</td>
<td>TYG-RGD-TRN</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>$2.0 \times 10^{-10}$</td>
<td>$3.5 \times 10^{-10}$</td>
<td>NI</td>
<td>PIP-RGD-WRE</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>$1.0 \times 10^{-10}$</td>
<td>$1.0 \times 10^{-10}$</td>
<td>NI</td>
<td>SFG-RGD-IRN</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>$2.5 \times 10^{-10}$</td>
<td>$2.5 \times 10^{-10}$</td>
<td>NI</td>
<td>TWG-RGD-ERN</td>
<td></td>
</tr>
</tbody>
</table>

Ability of the recombinant antibodies to block vitronectin binding to $\alpha_\beta$ and $\alpha_\beta$ and fibrogenin binding to $\alpha_{IV}\beta_1$ was determined with a purified receptor binding assay as described (20–22). The sequence of each recombinant CDR3 was determined by dideoxynucleotide sequencing. The sequences flanking the RGD motif of each antibody are shown. NI, no inhibition at concentrations of up to $5 \times 10^{-7}$ M.
Fig. 2. Fab-9 inhibits β3 integrin function by mimicking its ligand. (A) The ability of recombinant Fab-9 to block ligand binding to α5β1, αvβ3, and α5β3 was determined with a purified ligand–receptor binding assay (20–22). 125I-labeled vitronectin (Vn) was the ligand for α5β1 (○) and αvβ3 (○) and 125I-labeled fibrinogen (Fg) was the ligand for α5β3 (●). The inhibition assay was performed at saturating ligand concentrations. (B) Fab-9 blocks cell adhesion to vitronectin but not to fibrinectin, as assessed with M21 melanoma cells. The vast majority of the adhesion of these cells to vitronectin is mediated by α5β1 (45). The framework antibody anti-gp120 had no effect on cell adhesion to either vitronectin or fibrinectin. (C) Fab-9 mimics ligand by supporting cell adhesion as determined by immobilizing Fab-9 or vitronectin in microtiter wells at the given concentrations. Cells were allowed to adhere to immobilized ligand for 40 min. Nonadherent cells were removed by washing and adherent cells were quantified with an colorimetric assay for acid phosphatase (23). (D) Fab-9 binds to the RGD binding pocket of β3 integrins. The binding of the 125I-labeled Fab-9 to purified α5β3 was challenged with peptides GRGDSP or GRGESP. The latter peptide, which contains the substitution of E for D, cannot bind α5β3 and has no effect on the binding of Fab-9 to receptor. (E) The divalent-ion dependence of 125I-labeled Fab-9 to α5β3 was assessed in the purified receptor binding assay. Immobilized α5β3 was depleted of endogenously bound divalent ions by treatment with EDTA. The receptor was replenished with 0.5 mM Ca2+, 0.5 mM Mn2+, or 0.5 mM Mg2+. Each of these ions was able to support Fab binding to the receptor. Nearly identical results were obtained with αvβ3 (data not shown).

found in any known ligand. All purified Fabs compete with vitronectin, the natural ligand, for binding to the receptor with subnanomolar IC50 values (Table 1). The most potent snake venoms compete in the nanomolar range. The affinity of Fab-9 was further characterized by surface plasmon resonance to yield a Kd value of 2.5 × 10^{-10} M (34).

There are three major criteria for a probe of an integrin ligand binding site: (i) the antibody must block the binding of the natural ligand for the receptor, (ii) RGD peptides should block the binding of the antibody, and (iii) the binding between antibody and integrin should be divalent-cation-dependent. Of the series of recombinant antibodies selected through panning, Fab-9 had the highest affinity. Hence, we tested its ability to meet these criteria. We also tested the specificity of this antibody by assessing its reactivity toward related integrins. Integrins α5β3 and αvβ3 have several macromolecular ligands. Fab-9 blocked the binding of vitronectin, fibrinectin, and fibrinogen to both receptors. Data shown in Fig. 2A illustrate the ability of Fab-9 to block the binding of the highest-affinity ligand for each receptor, vitronectin–α5β3 and fibrinogen–αvβ3. In the same assay, all of the recombinant antibodies generated in this series failed to block vitronectin binding to α5β3 with any appreciable affinity. This is a striking display of specificity since this integrin contains the αv subunit and a β subunit 55% identical to β3 (35, 36). Since small RGD peptides have an equally high
affinity for αβ and αβ (data not shown), the appropriate display of this sequence within the CDR effectively programmed receptor specificity into the antibody. The HCDR3 of a mouse monoclonal antibody has been utilized previously to express a foreign sequence or epitope within the architecture of an antibody to produce antigened antibodies (37). These antibodies, however, are not functional with respect to binding another antigen and serve only as a vehicle for their display. Recently, this approach has been demonstrated to stimulate T-cell immunity (38). It is interesting to notice that several murine monoclonal antibodies have been prepared by immunization with αββ (46, 47). These antibodies generally contain an Arg-Tyr-Asp sequence in HCDR3. Furthermore, these murine antibodies bind with 2- to 342-fold lower affinity to αββ as compared to the synthetic human antibodies described herein.

As a more robust challenge to the potency of Fab-9, we examined its ability to block cell adhesion. In cell-based assays, receptor clustering can increase cellular avidity and cells have the potential to metabolize inhibitors. These conditions can override the effects of a weak inhibitor. Fab-9 blocked melanoma cell adhesion to immobilized vitronectin with an IC50 value of 4 nM (Fig. 2B). The framework antibody hiv12 had no effect on cell adhesion. More importantly, Fab-9 did not interfere with adhesion to vitronectin, an event largely mediated through β integrins. This finding provides further evidence that Fab-9 is specific to β integrins. In a similar way, we compared the adhesive capacity of Fab-9 to that of vitronectin. Both proteins supported cell adhesion. Vitronectin was only slightly more effective (Fig. 2C). The binding of Fab-9 to purified αββ (Fig. 2D) and αββ (data not shown) is competed by RGD peptides but not by the inactive RGE analog. The binding of this antibody to purified receptor is also divalent-cation-dependent (Fig. 2E). Collectively, the data indicate that Fab-9 binds the ligand binding pocket of αββ and αββ with high affinity and specificity.

Antagonists of these receptors, such as Fab-9, may be of utility in the treatment of osteoporosis and as antimitastatic (39–41) and antithrombotic agents. The semisynthetic anti-integrin antibodies could also have several research applications. Currently, the best method to elucidate the in vivo function of an integrin is to produce transgenic mice that have the gene ablated. Unfortunately, if the receptor has a crucial role in early development, only limited information can be gained from this approach. Antibodies may provide a way of determining receptor function in vivo and would conceivably mask potential side effects of anti-integrin therapeutics.

This methodology is not necessarily limited to integrins, as naturally occurring antibodies have been described that mimic many ligands. F42). The minimal binding sequences of a number of receptors have been described or are accessible through the screening/selection of linear peptide libraries (43, 44). Such sequences might be optimized in CDR regions as described above to endow the peptides with the desirable pharmacokinetics of antibodies as well as to further optimize their binding characteristics. In many cases, such human antibodies may find direct utility as drugs, diagnostic agents, or vaccine vehicles. Furthermore, the speed with which these reagents were generated exceeds even animal immunization. This approach may also find application in drug design. The optimized CDR loop can be viewed as a conformationally constrained peptide that serves as a lead compound (4). The structure of the loop is accessible as methodologies for the structural prediction of CDRs are well developed (29, 30), and more importantly, Fab fragments are amenable to crystallographic investigation. As such, ligand-mimicking antibodies may prove to be useful surrogates when structural investigation of the natural ligand is intractable.

Note Added in Proof. Subsequent to the preparation of this manuscript we have examined the applicability of the method to discover other binding motifs. In this case, the RGDX sequence within the optimized context of Fab-9 was randomized and rescreened for binding. The clones that resulted contained additional non-RGD motifs and demonstrated improved affinity and receptor specificity (C.F.B., Dana Hu, and J.W.S., unpublished data). The characterization of these antibodies will be reported elsewhere.

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