To the editor:

The limitations of site-directed mutagenesis in the localization of Rh D epitopes

Recently Liu et al used elegant and extensive experiments to determine the precise polymorphisms in the Rh D amino acid sequence that differentiate binding by a cohort of antibodies directed against different Rh D epitopes.1 But the authors asserted that “their evidence directly refutes the recent hypothesis of Chang and Siegel.”2(p3998) This erroneous claim arises from an overinterpretation of their results and a misunderstanding of our model.

There are at least 37 accepted Rh D epitopes, as defined by differential agglutination of Rh D variant red blood cells. Classically, these epitopes are believed to cover different regions of the Rh D molecule. We hypothesized that most, if not all, Rh D epitopes differ, not spatially, but only in the number and arrangement of their potential contact residues. That is, the footprints of most anti–Rh D antibodies are essentially identical to one another.

Liu et al used site-directed mutagenesis to express Rh D variant molecules genetically intermediate between wild-type Rh D and the highly homologous Rh cE molecule. Of the approximately 100 exposed amino acids, 9 polymorphisms define the difference between Rh D and Rh cE. They determined the binding patterns of a panel of anti–Rh D antibodies after substituting in various combinations of Rh D residues onto the Rh cE backbone. Based upon which mutations were necessary to reconstitute binding, they defined “distinct local epitope clusters” against which they believe the antibodies bound.1(p3989)

We believe that the latter conclusion is an overinterpretation of their data. For example, in their discussion of the loop 6 cluster comprising 3 polymorphisms, the authors very carefully state that “no other D-specific amino acids are required for D epitope expression.”1(p3990-3991) The authors did not exclude the existence of any interactions between their anti-epD3 antibodies and the 91 residues that are conserved between Rh D and Rh cE. Based upon the crystallographic studies of the model anti–hen egg lysozyme antibody, D1.3, and the antineuraminidase antibody, NC41, a high-affinity antibody may directly contact anywhere from 14 to 22 different residues on the corresponding antigen and may comprise over 30 hydrogen bonds.3 Thus we believe it highly unlikely that the interactions between Rh D and anti–Rh D antibodies are confined to just the polymorphic amino acids studied.

This interpretive issue was illustrated in Figure 10B in our paper, where we proposed an alternative model for understanding the nature of the serological reactions that define wild-type Rh D and Rh D variant VI molecules. The Rh D polymorphisms differentiating wild-type Rh D and variant VI were represented by an asterisk (ie, the ledge). Note that the mutation of variant VI with wild-type Rh D residues results in the removal of the ledge and the gain of binding by an anti-epD2 antibody. By Liu et al’s criteria, the mutatedledge defines the “distinct local epitope cluster” where the anti-epD2 antibody binds. Their definition of the epD2 epitope would not include other interactions, such as those on the left where the anti-epD2 antibody interacts with residues conserved on wild-type Rh D, Rh D variants, or even Rh cE molecules. Furthermore, due to the highly conformational nature of Rh proteins, it may not be correct to assume that residues shown to be responsible for the expression of certain epitopes necessarily are the ones that serve as contact points for their respective antibodies. For example, G96S and L245V mutations in the protein encoded by the RHCE gene affect the expression of Rh(c) (and Rh26) and the Rh VS antigen, respectively.10,11 But these residues, located within the third and eighth transmembrane segments, respectively, cannot obviously serve as sites for antibody binding.

Thus Liu et al’s experiments did not “localize” the epitope, as they claim. Instead, they determined which of the 9 Rh D polymorphisms are necessary for binding and which were not. Site-directed mutagenesis is a powerful tool, but as Greenspan and Di Cera recently noted,4 one needs to be cautious about overinterpreting the resulting data. Furthermore, Liu et al’s experiments are completely consistent with our hypothesis of overlapping footprints. Of note, their results are also consistent with the classical model as depicted in our Figure 10A; therefore, their data, though elegant, fail to address our “identical footprint” hypothesis.

Prior analysis of model antibody-protein antigen complexes revealed that the corresponding epitopes spanned areas of 748-878 Å².2 In contrast, the extracellular surface of Rh D is estimated to be approximately 50 Å in diameter (about 2000 Å²).1 Although the calculation of extracellular surface underestimates the potential area of interaction, this fundamental constraint limits the number of spatially distinct epitopes that can fit onto the surface of Rh D.

In our manuscript, we made the surprising observation that the amino acid sequence and epitope specificity of a large cohort of anti–Rh D antibodies did not segregate cleanly, as would be expected for spatially and structurally distinct epitopes. Instead, these antibodies, directed against 1 novel and 4 of the 6 major Rh D epitopes, were remarkably related genetically irrespective of specificity. For example, the VH3-33 VH germline gene could give rise to antibodies recognizing any of the 5 major epitopes, and one anti-epD2 antibody differed from an anti-epD3 antibody by only 3 amino acids (97% complementary determining region [CDR] aa homology; 99% Fv [variable region] homology). This implied that the paratopes of antibodies against different Rh D epitopes could be highly similar. In contrast, the antineuraminidase “counterexample” quoted by Liu et al compared the binding of 2 genetically unrelated antibodies with 35% aa homology in the CDRs and 53% homology in the Fv.5

Given the spatial constraints of the Rh D antigen and the genetic constraints of our antibodies, we proposed that antibodies against different Rh D epitopes define differences in fine specificities, rather than spatially distinct epitopes. This hypothesis was validated by cross-competition experiments: independent of epitope specificity, our anti-Rh D antibodies inhibited each other’s binding. Others have since demonstrated the ability for a single monoclonal anti-Rh D to block polyclonal anti-Rh D binding.6,7 These findings are clearly inconsistent with the notion of spatially distinct epitopes but are also distinct from a claim for “the entire external surface of the D protein to encompass 1 single binding site,” for which we were falsely attributed.1(p3993)

How can the concept of “identical footprints” be reconciled with the differential agglutination of Rh D variant cells? As illustrated in our model, the Rh D antigen likely makes multiple contacts with an antibody. Mutations that give rise to anti–Rh D antibodies modify the potential interactions by gaining or losing points of interaction. Antibody mutations that disrupt contact points can prevent antibody binding; conversely, mutations that increase the
number or strength of interactions will increase the affinity of the antigen-antibody interaction, converting a negative reaction into a positive binding result. Because Rh D and its variants present slightly different contact points, differential binding will occur when wild-type Rh D presents sufficient contacts for binding, but variant Rh D does not. This model is analogous to the interaction between mutants of the antibody 26-10 with digoxin, where the P96Y and P96F mutations sterically interfere with the hydroxyl group on digoxin resulting in loss of binding but do not interfere with the hydroxyl-free digitoxin, which maintains high-affinity binding.8

Since our report, Hughes-Jones et al derived several pan-agglutinins and anti–Rh E antibodies from anti–Rh D antibody genes.9 Their pan-agglutinin antibody differed by only 10 amino acids from anti-Rh D specific antibodies. Of their 12 anti–Rh E specific antibodies, 3 used identical heavy- and light-chain germ-line genes (VH, JH, VL, and JL), as did our anti–Rh D specific antibodies. Because the “sequence space” of all possible amino acid sequences was not exhaustively examined, these studies provide an upper limit on the minimal genetic difference between anti–Rh D and anti–Rh E specific antibodies.

Thus anti–Rh E specific and anti–Rh D specific antibodies are genetically very close and, thus, likely share highly similar paratopes. Hughes-Jones et al’s results were highly satisfying with regard to our “identical footprint” model: because Rh D and Rh cE are so highly homologous, it was not surprising that antibodies directed against Rh D and Rh cE antigens be closely related genetically. As with the Rh D epitopes, we believe that for many antibodies the specificity differentiating Rh D, Rh E, and other Rh-related epitopes may be dependent upon the number and arrangement of the specific contact residues presented.

Response:

Limitations of prediction of epitope structure from corresponding antibody sequence

We welcome the dialogue invoked by Drs Chang and Siegel. The identification of Rh D epitopes is important in analyzing immune responses to the D antigen and gives insight into its structural characteristics. Two models argue for the molecular configuration of D epitopes: our cluster model,1 under which we propose there are at least 6 distinct regions (clusters) of the Rh D protein involved in epitope presentation; and Chang and Siegel’s “identical footprint” model,2 which predicts all anti-D paratopes bind to a similar footprint on the Rh D protein, differing only in the numbers and positions of contact amino acids. The 2 models differ in that we suppose that there are multiple anti-D footprints, some as small as a single external loop of the protein with others comprising 2, 3, or even 4 external loops, whereas Chang and Siegel suppose that there is just 1.

We disagree that we overinterpreted our data (we had emphasized the limitations of our work) and that we have misunderstood their model. We had fully acknowledged that “true” epitope mapping would entail analysis of anti-D/Rh D protein crystals to define the identities of all amino acid contacts. Site-directed mutagenesis (SDM) studies are acknowledged to be extremely powerful, albeit with some limitation, in particular, when epitopes are disrupted. Chang and Siegel inaccurately cited Greenspan and Di Cora,3 in order to justify their claim that our approach was not accurate in the definition of D epitopes. Greenspan and Di Cora describe the limitations of alanine scanning mutagenesis (ASM) as applied to epitope mapping, not an approach we adopted. We rejected such an approach because disruption of D epitopes may have undefined effects of Rh D protein conformation.11 As we have stressed, we “created” D epitopes and did not disrupt them (as ASM would do). This was done by creating chimeric Rh cE→D constructs, where exposure of key Rh D amino acids on essentially a Rh cE protein gives considerable insight in how these residues participate in D antigenicity. Our studies do indeed refute the “identical footprint” model of Chang and Siegel: there are spatially distinct D epitopes, and this is backed by irrefutable immunochemical evidence. We argue that our data provide direct evidence for the involvement of particular Rh D amino acids in expression of D epitopes. Chang and Siegel have speculated on the nature of D epitopes based solely on the sequences of their corresponding anti-D paratopes. This assessment cannot predict their molecular origins with any accuracy and is highly speculative. We confined our speculation to assimilation of our data with that available on the molecular biology of Rh antigen expression. We believe that the presentation of hypothetical models is a powerful tool in contemporary biology, and we presented a model for the loop-to-loop interactions of a Rh D protein monomer. The model proposed initially by Chang and Siegel was indeed elegant and provided a strong impetus to develop our experimental approach.

We did not argue that “interactions between Rh D and anti–Rh D antibodies are confined to just the polymorphic amino acids studied.” Of the relatively limited number of antibody-antigen complexes resolved by crystallography (cited in our paper), all involve the contact of at least 15 amino acids. Our model states that “loop 6-dependent epitopes require only an RHD loop 6 for antibody binding.”123 As this external loop is predicted to be approximately

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16-23 amino acids, we speculated that this loop constitutes this binding site for anti-D epitopes with this epitope specificity, but critically involving the D-specific amino acids 350, 353, and 354. At no point did we ever suggest that just 3 Rh D amino acids are sufficient for antibody binding, but clearly they are critical, either forming some of the contact residues or altering the configuration of the Rh D protein to an extent to permit antibody binding. Furthermore, our D epitope cluster model presented in Figure 4 clearly suggests that overlapping epitope clusters, requiring one or more Rh D protein loops are required for antibody binding. Our work will therefore provide information regarding D epitope–critical amino acids when crystals of anti-D binding to Rh D proteins become available.

Furthermore, Chang and Siegel have conveniently overlooked other data that undisputedly argue for distinct epitopes on the Rh D protein. An anti-D that has immunoblot activity has been identified (LOR-15C9), and it requires loop 6 Rh D residues 350, 353, and 354. In this instance, only one explanation exists for the binding site of this antibody: it is a continuous peptide sequence on the RhD protein, and any conformational requirement of the Rh D protein has been disrupted during SDS-PAGE. Most anti-D paratopes are highly conformation-dependent and do not immunoblot. Our model is entirely consistent with the existence of this antibody, and its existence refutes the argument that all anti-D have a common footprint. In addition, several groups including ours have produced rabbit Rh antipeptide polyclonal antisera corresponding to external loops of the Rh D protein. These antisera react with native Rh polyepitides and are, thus, directed against short linear (16 amino acids or less) stretches of Rh protein sequence as defined by the immunogen. Thus the paratopes involved in epitope binding are comparatively small and cannot be a large conformation-dependent epitope necessary to provide a large footprint of anti-Rh binding. If the rabbit repertoire is capable of responding to a short Rh D peptide sequence, then there is no reason why human B cells cannot do the same; antibodies such as LOR-15C9 illustrate that this is indeed the case.

A further compelling argument for our theory for distinct D epitope clusters was the physical size of the 6 Ig complementary determining regions (CDRs) compared to the external face of the Rh D protein monomer. We predicted that the Rh D protein has a diameter of no less than 50 Å by comparison to crystal structures of known dimensions (aquaporin 1 and band 3). Immunoglobulin paratopes do not exceed 20-25 Å in diameter. Paratopes have a variety of different surface areas of contact dependent on the 3-dimensional configuration of its corresponding epitope. Thus there is a physical constraint on the size of Rh D epitopes that can actually be. Because loss of D epitopes can be associated with mutations on every loop of the RhD protein (as defined by partial D phenotypes), it is extremely unlikely that a single footprint can straddle the complete external surface of the Rh D protein to encompass all 6 loops. This fundamental observation is consistent with structurally distinct epitopes but entirely inconsistent with a single footprint (see Figure 4 in Liu et al1 and Figure 4 in Avent10). The argument that cross-competition experiments rule out the possibility of spatially distinct epitopes is flawed. Anti-D binding sites are at the very tip of immunoglobulin molecules, the Fc portion of which provides a huge steric constraint to the approach of further anti-D and/or recombinant phage to their epitopes.

Chang and Siegel defined the epitope specificity of their anti-D Fvs using partial D phenotype red cells. They concluded that they have antibodies with anti-epD2 and -epD3 specificity, based on their reaction profiles. But the “anti-epD3” classification they have used is inaccurate. Because they did not use Dm phenotype red cells, they cannot differentiate whether their antibody is of anti-epD3 or -epD9 specificity. The “old” 9-epitope model classifications of epD3 and epD9 encompass a wide variety of anti-D epitopes with different reaction profiles (as demonstrated by the differences in reactions observed in the 10 anti-D of this specificity that we analyzed) and is extremely difficult to define using just 6 partial D variants, as done by Chang and Siegel. Nevertheless, we are not surprised that 3 amino acid differences in an anti-D sequence can invoke such differences in antibody binding: some anti-epD2 and all anti-epD3/9 have binding sites on the sixth loop of the Rh D protein.1 EpD2 also contacts residues on loops 3 and/or 4 of the Rh D protein, with this interaction being disrupted on D31 phenotype red cells (equivalent to the “ledge” in Chang and Siegel’s model). Our model for epD2/3 expression is not dissimilar to Chang and Siegel’s: both of these epitopes overlap but do not correspond to the “conventional” model proposed by Chang and Siegel (Figure 10A), which suggests that these epitopes are physically separate. Our argument is that the footprint for epD2 binding is essentially larger than that of some epD3 and that the dimensions of anti-D paratopes and D epitopes proposed by Chang and Siegel in their model (Figure 10B) are inaccurate. Chang and Siegel have chosen to highlight the close genetic relationship between and anti-epD2 (E1/M2) and an anti-“epD3” (E1/M3) to drive their argument for a single footprint of anti-D binding, but they have identified significant differences between the heavy- and light-chain CDR sequences in other anti-D: this is entirely consistent with structurally distinct epitopes. We believe that the generalization that “anti-Rh E specific and anti–Rh D specific antibodies are genetically very close and, thus, likely share highly similar paratopes” is misleading. We have no doubt that some anti-D epitopes share similar paratopes to some anti-E epitopes, presumably because they both involve binding sites that include loop 4 of the Rh D and Rh E proteins, respectively. The close genetic relationship between some anti-D epitopes and anti-E epitopes vindicates our argument that just small differences in anti-Rh CDR sequences can invoke binding to spatially distinct epitopes, in this instance, on 2 completely different protein species.

To the hematologist and transfusion scientist, the rather bewildering classification of D epitopes can appear superfluous. But our study has allowed the prediction of loop-to-loop interactions of the Rh D protein, which may indicate which regions of the protein are most immunogenic. This model was constructed on the understanding that Rh D protein loops can be no more than 25 Å apart in order to contact the paratope of an anti-D epitope. Our work has provided direct evidence of the molecular requirements for D antigenicity. We believe that the prediction of D epitope structure from sequencing a limited number of anti-D epitopes is unproductive and must take account of the emerging information now available concerning the molecular biology of Rh antigenicity.

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To the editor:

A potential role for leukemia inhibitory factor in the increased clonogenicity of human fetal progenitor cells

A recent report by Shih et al demonstrates the ex vivo expansion of human fetal CD34<sup>+</sup>, thy-1<sup>-</sup> hematopoietic progenitors in the presence of a leukemia inhibitory factor (LIF)–induced, murine stromal cell–derived factor. The authors show that expanded progenitor cells retain their immature immunophenotype in vitro and that the engraftment capacity in murine transplantation models is preserved. In this and their previous report, they allude to the potential utility of such an expansion promoting factor (SCEPF) in the clinical arena, including the transplantation of stem cells from cord blood.

In their experiments, a given LIF concentration of 10 ng/mL results in the production of a LIF-induced expansion promoting factor by the murine stromal cell line AC 6.21. They do not report how this concentration was determined to be optimal, nor do they report whether a dose response relationship between LIF concentration in AC 6.21 stromal cell culture and the magnitude of expansion was observed. The experimental design did not seem to address a potential direct effect of human LIF on the augmented production of SCEPF in murine stroma, human LIF used at nanogram concentrations has an independent effect on the expansion of human fetal progenitors. Taken together, the augmented production of LIF by cord blood mononuclear cells may directly and/or indirectly underlie the increased in vitro and in vivo clonogenicity of cord blood, compared to adult PBMC.

We therefore wish to suggest that the expansion effect described by Shih et al may rely on the supraphysiologic levels of LIF in their stromal cell culture system and that in turn lead to the elaboration of an expansion promoting activity. Moreover, it remains to be determined, whether in addition to the induction of SCEPF in murine stroma, human LIF used at nanogram concentrations has an independent effect on the expansion of human fetal progenitors. Taken together, the augmented production of LIF by cord blood mononuclear cells may directly and/or indirectly underlie the increased in vitro and in vivo clonogenicity of cord blood, compared to adult PBMC.