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Monoclonal Antibodies Specific for Discontinuous Epitopes Direct Refolding of Influenza A Virus Hemagglutinin

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Abstract

Antibodies (Abs) specific for the globular domain of influenza A virus hemagglutinin (HA) efficiently neutralize viral infectivity and provide the most effective protection against influenza following infection or vaccination. Nearly all neutralizing Abs recognize discontinuous determinants formed by residues present on different stretches of the HA primary structure. Here, we show that approximately 25% of a large panel of neutralizing monoclonal Abs (mAbs), including Abs specific for each of the four major antigenic sites, can bind to completely denatured HA. Binding of these mAbs to denatured HA occurs much more slowly than binding to native HA, but bound mAbs dissociate from denatured and native HA with similar kinetics, and amino acid substitutions that reduce mAb binding to native HA have a similar effect on mAb interaction with denatured HA. HA refolding induced by mAb binding facilitated the binding of mAbs to other antigenic sites, indicating that refolding was not limited to the antibody-interaction domain. These findings validate the localization of antigenic sites by identifying amino acid substitutions selected in mAb escape mutants. Further, they demonstrate that Abs can facilitate the refolding of denatured proteins, which suggests a number of practical applications for optimizing antibody based assays, and also for potentially using Abs as specific chaperones for protein refolding.

Introduction

The influenza A virus (IAV) hemagglutinin (HA) is a viral surface receptor glycoprotein of great medical and scientific significance. HA initiates the infectious cycle by attaching virions to sialic acid receptors on host cells and mediating fusion of viral and host membranes (Skehel and Wiley, 2000). Antibodies (Abs) to HA block virus attachment or fusion and neutralize viral infectivity (Gerhard, 2001). Of myriad immune effector functions, only Abs can provide complete protection against IAV infection. Due to antibody pressure in humans, IAV demonstrates constant antigenic evolution that thwarts development of a vaccine that is effective for more than a few years (Air et al., 1987). As a result, IAV remains a significant cause of mortality and morbidity throughout human populations (Fauci, 2006).

Due to its medical significance, HA has been intensively studied for decades. Among many other firsts dating back to the 1930's, HA was the first protein to be intensively investigated with monoclonal Abs (mAbs) (Yewdell and Gerhard, 1981), the first viral protein to be structurally characterized by x-ray crystallography (Wiley et al., 1981; Wilson et al., 1981), and the first protein shown to induce membrane fusion (Skehel et al., 1995). Due to this

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foundation of knowledge, HA serves as a model protein for understanding viral attachment, fusion, antigenicity and antigenic escape.

HA is a trimeric protein that forms a globular domain containing the sialic acid binding site sitting atop an extended stalk (Gamblin et al., 2004). A detailed antigenic map of the A/Puerto Rico/8/34 (PR8) HA was generated by using mAbs to select a panel of spontaneously arising escape mutants of which ~ 50 possessed unique amino acid substitutions, the vast majority due to point mutations (Caton et al., 1982). Mapping of the substitutions onto the 3-dimensional structure revealed the presence of 4 distinct antigenic sites in the globular domain (Figure 1), termed Sa (red), Sb (blue), Ca1 (orange)/Ca2 (olive), and Cb (teal). Each of the antigenic sites comprises residues derived from different stretches of primary sequence. The Ca sites bridge adjacent monomers, and the binding of some Ca specific mAbs is dependent on HA trimerization during biogenesis.

Most immunological assays are based on the binding of Abs to uncharacterized and probably highly heterogeneous forms of target antigens. Only in function based assays (e.g. virus neutralization, hemagglutination inhibition) is it certain that the relevant form of antigen is in native or near native state. In the present study, we examine the ability of anti-HA mAbs with well characterized epitopes to bind unfolded HA. Our findings have important implications for understanding antibody-antigen interactions in common technologies such as ELISA and immunoblotting and raise the possibility of a novel application of mAbs in directed protein folding.

Materials and Methods

Virus and radioimmunoassays

All experiments used *wt* or previously described mAb selected escape mutants of A/Puerto Rico/8/34 (H1N1) IAV. Virus was grown in the allantoic sac of 10 d embryonated chicken eggs and purified by velocity centrifugation in sucrose gradients. Purified virus was denatured by adjusting virus PBS at 150,000 hemagglutinating units (HAU) per ml to 1% w/v DSD, 300 mM Tris HCl pH8, 10 mM DTE, and incubating for 5 min in a boiling water bath. After chilling to 0 °, freshly prepared iodoacetamide was added to a concentration of 15 mM, and the alkylation reaction was allowed to proceed in the dark for 10 min until stopped by the addition of DTE to a concentration of 22 mM. Denatured virus was used within a few hours to prepare RIA plates. In initial experiments, we removed denaturants from viral proteins by G25 sephadex chromatography. When later experiments revealed that this did not alter the ability of denatured proteins to interact with mAbs in the RIA this step was eliminated. Before their use in IRAs, plates coated with immunoabsorbents were washed several times with PBS and incubated for a few hours with PBS containing 1% BSA (w/v) at 4°C to block residual protein binding sites. After 0, 24, 48, and 70 hrs additional incubation, PBS-BSA was replaced with 25 µl hybridoma tissue culture supernatants diluted 1/4 in 1% BSA-PBS. After 72 h, all Abs were removed and well washed 4 times with PBS. The amount of bound mAb was then determined by adding 25 µl PBS-BSA containing 100,000 cpm radioiodinated rat anti mouse κ chain Ab. Following 2 h incubation at 4°C, wells were washed with PBS and the amount of bound Ab determined by γ -counting.

mAbs

All mAbs used in this study have been described in previous publications (Caton et al., 1982; Gerhard et al., 1981; Yewdell et al., 1986; Yewdell et al., 1981; Yewdell and Gerhard, 1981; Yewdell et al., 1993; Yewdell et al., 1979). mAbs were used a hybridoma culture fluid supernatants.

Direct RIAs

Y8-10C2 was purified by affinity chromatography using protein-A sepharose and radioiodinated using the chloramine T method to a specific activity of 2×10^7 cpm/ μ g Ab. The fraction of function labeled antibody was determined to be 75% by sequentially incubating labeled Ab with native polyvinyl bound virus (or BSA as a binding control) until all binding activity was exhausted. For affinity measurements, Y8-10C2 was incubated at 4 °C with native or denatured virus adsorbed in polyvinyl 96 well plates and washed before bound Ab was determined by γ -counting. Background binding was estimated by incubating antibody with wells coated with BSA and subtracted from binding values. Data were analyzed using GraphPad Prism software and the association constant was estimated using the One Binding Site curve fitting algorithm, $\text{Bound} = (\text{Max Binding} \times [\text{input}] / (K_D + [\text{input}]))$.

Results

Immunogenicity and antigenicity of denatured IAV proteins

Over the years, Walter Gerhard and colleagues produced numerous HA-specific mouse mAbs by screening hybridoma supernatants in indirect radioimmunoassay (RIAs) against purified IAV or IAV-infected cells absorbed to polyvinyl 96 well plates (Yewdell and Gerhard, 1981). Out of hundreds of mAbs generated from mice immunized with live virus or infected cells, only one defined anti-HA mAbs, H18-L10 failed to interact with native HA as inferred from complete absence of virus neutralization or hemagglutination inhibition activities. Tellingly, when tested for binding to purified IAV that was denatured by boiling in SDS and alkylated with iodoacetamide to prevent reformation of disulfide bonds prior to adsorption to polyvinyl (Table I), out of more than 100 of these HA specific mAbs tested (including the Sb specific mAb H28-E23), only H18-L10 demonstrated robust binding.

H18-L10 is specific for the HA2 subunit of HA, which is present in the stalk of the HA and is sterically inaccessible to antibody binding on intact virions (Yewdell et al., 1993). Consistent with its ability to bind denatured HA, H18-L10 exhibits a greatly enhanced ability relative to nearly all HA1-specific mAbs to detect HA in immunoblotting after antigen is boiled in SDS with or without reduction of disulfide bonds (Yewdell et al., 1993). When denatured virus was used to generate hybridomas screened by RIA using denatured virus bound to polyvinyl, each of the 4 high binding mAbs that were characterized (Table I: RA4–44, RA5–22, RA6–30, RA8–57) were found to bind to HA2, as determined by immunoblotting (data not shown). Thus, HA2 retains immunogenicity and antigenicity in a denatured state.

HA2 is cleaved from HA1 in the egg grown virus used for RIA, so it was possible that the failure of HA1-mAbs to bind to denatured virus was due to the lack of binding of denatured HA1 to polyvinyl in a form accessible to antibody. This is clearly not the case, however, since CM1–4, a mAb raised to a synthetic peptide corresponding to HA1 residues 111–119 (Eisenlohr et al., 1988), binds well to denatured virus under standard assay conditions (Table I).

Similarly, the failure to identify hybridomas specific for NP or M1 from mice immunized with denatured virus cannot be attributed to the lack of binding of denatured NP or M1 to polyvinyl (Table I, II). M1 specific mAbs interact well with denatured M1 (as determined by this RIA and also by immunoblotting), so the absence of M1 mAb is probably due to the poor overall immunogenicity of M1 in mice. By contrast, NP is highly immunogenic in mice, as NP specific mAbs are commonly observed in hybridomas generated from mice immunized with live virus or infected cells (Caton et al., 1991; Yewdell et al., 1981). Like HA1, however, nearly all NP-specific Abs behave like H16-L10 (and not H19-S24, Table I), interacting poorly with denatured NP in RIA or in immunoblotting (data not shown). The extent to which this is due

to the dependence of the corresponding epitopes on NP tertiary structure vs. oligomerization remains to be determined. It is worth noting that all of dozens of NP-specific mAbs characterized bind well to NP expressed by recombinant vaccinia virus, and therefore do not depend on the interaction of NP with other IAV gene products or IAV-induced post-translational modifications in NP (data not shown)..

Based on these findings we conclude that denaturation of HA greatly reduces the affinity of neutralizing HA specific Abs and favors the immunogenicity and antigenicity of HA2 specific Abs. This is not surprising given the globular nature of the HA receptor binding domain and the extended structure of its stalk, which is essentially a bundle of extended α -helices (Figure 1).

A subset of native HA specific mAbs bind to denatured HA with slow kinetics

Antibody binding is capable of altering antigen conformation. This is most clearly shown with enzymes, where Abs can *enhance* enzyme activity (Melchers and Messer, 1970). We reasoned that given sufficient time, Abs against native HA might bind and be able to freeze a denatured epitope in the proper conformation as the denatured antigen visits different conformational states. To test this idea, we absorbed native or denatured virus to polyvinyl and then incubated with a panel of mAbs for increasing times. While native HA specific mAbs binding reached near maximal values within 2h, the binding of a number of native-HA specific mAbs to denatured virus increased throughout a 3-day incubation (Figure 2). By contrast, the denatured HA specific mAb CM1-4 demonstrated exactly the opposite behavior; binding rapidly to denatured HA and increasing its binding to native virus with time. Note, however, that binding of CM1-4 to native HA was at a higher level at 2 h than binding of native-specific mAbs to denatured HA. This indicates that a significant amount of denaturation accompanies HA binding to polyvinyl.

The time dependent binding of native-specific mAbs to denatured HA is not due to stable refolding of the HA during the course of the assay. This conclusion follows from the protocol employed: reactions were initiated sequentially and ended simultaneously using a single set of RIA plates. Thus the slow binding must be reflection of a low on-constant for mAb binding to denatured HA.

Having established the time-dependent nature of native HA specific mAb binding to denatured HA, we tested a panel of 70 mAbs for their ability to bind denatured HA after 5 day incubation. While all of the mAbs bound at high levels to native HA, 23 (16%) bound at significant levels above background values to denatured HA. Included in this set were multiple Abs specific for epitopes mapping to each of the 4 antigenic sites.

Recognition of denatured HA is based on epitope refolding

The interaction between Abs and protein antigens typically encompasses ~ 20 residues between both binding partners (Wilson and Cox, 1990). It is clear however, that a more limited area of interaction can still result in high avidity binding. To cite a cogent example, the CM1-4 mAb binds at high levels to its immunizing HA₁₁₁₋₁₁₉ peptide bound to polyvinyl. Although it is likely that most of the 23 denatured HA reactive-mAbs recognize discontinuous epitopes on native HA, it was possible that their interaction with denatured HA was based on binding a linear portion of the epitope.

To examine this possibility, we tested the binding of 5 representative mAbs to a panel of 8 escape mutants with defined amino acid substitutions (Table II). Similar amounts of denatured viruses were bound to polyvinyl as shown by binding mAbs specific for HA2, NP or M1. This experiment revealed that amino acid substitutions that reduce antibody binding to native HA

have a parallel effect on binding to denatured HA. Of particular note, mutations widely separated in the primary structure prevent the binding of Y8-10C2, H36-26, and H36-38. This is consistent with the conclusion that mAbs bind to epitopes that refold to recreate the spatial relationship of loops that form the native epitope.

Antibody induced refolding recreates the original epitope

We further characterized mAb interaction with denatured HA using radioiodinated Y8-10C2. As with unlabeled antibody, labeled antibody demonstrated increased binding with prolonged incubation (Figure 3 top). Although binding and calculated maximal binding increased with time, the calculated association constant (K_A) did not increase, and in fact decreased slightly. In a second experiment we directly compared binding of Y8-10C2 to native vs. denatured HA in a 20 h binding experiment (Figure 3 bottom). Y8-10C2 bound to native HA with a 10-fold higher K_A than binding to denatured HA.

If Y8-10C2 binds to a completely refolded epitope in denatured HA, then its off rate from denatured vs. native HA should be similar. We measured off rates by binding radiolabeled Y8-10C2 to native or denatured HA, washing, incubating wells with excess unlabelled Y8-10C2 (or a control mAb) to prevent rebinding of dissociated antibody and measuring amount of released label with time. Dissociation of Y8-10C2 from both denatured and native HA over a 24 hr period was negligible, strongly suggesting that the 10-fold decrease in affinity is largely if not entirely due to a decrease in its on rate.

These data indicate that Y8-10C2 binds to native and denatured HA with similar energy of interaction, and in conjunction with the escape mutant binding data, strongly supports the conclusion that mAbs recreate their native epitopes in denatured HA.

Antibody induced refolding extends beyond the renatured epitope

Since globular proteins typically fold in a domain specific manner, it was of great interest to examine whether mAb induced epitope refolding induced refolding of the non-overlapping epitopes. To examine this possibility, we incubated denatured HA (or native HA as a control) with various mAbs for 2 days and then measured binding to labeled Y8-10C2 (Table III). Two findings demonstrate the specificity and validity of this assay. First, unlabeled Y8-10C2 competed for binding of labeled Y8-10C2 to either native or denatured HA. Second, the Y8-1A6 mAb, which only binds native HA and recognizes an overlapping Sa epitope, competes for binding to native HA but not denatured HA. Other mAbs specific for Sb, Ca, or Cb epitopes had no effect on Y8-10C2 binding to native or denatured HA (data not shown).

The critical finding in this experiment is that refolding Sb or Cb epitopes by pre-incubating denatured HA with H36-26, or H33-23 mAbs, respectively, enhances binding of labeled Y8-10C2 (Sa-specific) by 3- to 6-fold. In contrast, neither mAb increased Y8-10C2 binding to native HA, demonstrating their ability to enhance Y8-10C2 binding to denatured HA is not based on a direct interaction between these mAbs and labeled Y8-10C2. Further, the HA2 specific antibody H18-L10 did not enhance radiolabeled Y8-10C2 binding to denatured HA. Thus, the mere binding of a mAb to denatured HA is not sufficient to enhance refolding of the Y8-10C2 epitope. Rather, this experiment strongly supports the conclusion that mAb induced local epitope refolding results in global refolding of the globular domain.

Discussion

After three decades of experience with anti-HA mAbs, it appears that nearly all Abs possessing virus neutralizing activity recognize conformational determinants created from residues derived from multiple loops of the primary structure (Knossow and Skehel, 2006). It is a

delicious irony that T cell determinants, which proved so vexing to establish following the discovery of MHC restriction, prove to be far easier to precisely define than antibody epitopes, whose definition ultimately requires x-ray crystallography of the mAb bound to its antigen. Even with the tremendous advances in crystallography related technology, this remains a laborious approach that succeeds for only a subset of mAbs.

The principal method for defining the epitopes of neutralizing mAbs to IAV, and dozens of other viruses, remains selecting neutralization escape mutants and using DNA sequencing to identify the mutations that reduce antibody affinity to the epitope. Interpretation is based on the assumption that the modified amino acids are present within the epitope and do not act at a distance. Although this assumption is probably correct in the majority of situations, there are examples of mutations acting at a distance to selectively modify a highly limited set of epitopes. For example, we showed that the Y8-10C2 epitope, which is rendered inaccessible by HA trimerization, is affected by mutations in that stalk that reduce the “breathing” of the globular domain (Yewdell et al., 1993).

Here we provide evidence supporting the validity of the escape mutation method of epitope mapping. We show that mAbs can facilitate HA refolding to reform their epitopes and that escape mutations modulate mAb binding to refolded and native HA to a similar extent. This approach is applicable to other viruses and antigens, but it is important to note that only a fraction of HA-specific mAbs can induce refolding. It will be of interest to correlate the refolding capacity of mAbs with their ability to interact with fragmented antigens expressed by phage display technology (Parmley and Smith, 1989).

The extremely slow kinetics of mAb induced refolding has an important practical application as it suggests that in immunoblotting and other denatured antigen based assays, Abs considered to be poorly reactive might demonstrate significant improvement if allowed to incubate with antigen for prolonged periods.

Our findings indicate that Abs have the potential to catalyze protein folding, much as they can catalyze enzymatic reactions (Benkovic, 1992). For this to be useful, it must be possible to dissociate the antigen-Ab complex in a manner that allows recovery of both components in biologically active forms. This could be achieved by screening mAbs or phage Abs for their ability to dissociate from antigen after mild changes in buffer composition (pH, salt, etc) or their dependence on divalent cations for binding antigen (Maurer et al., 1970). Existing Abs could be mutagenized and then screened for the desired binding characteristics. Abs able to catalyze protein folding could be of great use to the biotechnology industry, where it is most economical to produce mammalian proteins in unicellular organisms least able to fold proteins to their native biologically active states.

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Figure 1. Location of mAb Defined Epitopes on PR8 HA

Figure 2. Kinetics of mAb Binding to Native vs. Denatured Immunoabsorbant

The mAbs indicated were incubated for 2, 24, 48 or 72 h with native or denatured PR8 before washing and detection of bound antibody by addition of radioiodinated rat anti- κ chain mAb.

Kinetic Analysis of Y8-10C2 Binding

Y8-10C2 Binding to Native vs. Denatured

Figure 3. Binding Of Radiolabeled Y8-10C2 to Native or Denatured Virus

Y8-10C2 was radioiodinated and used to measure its affinity for denatured and native immunoabsorbents in direct RIAs. Dotted lines represent 95% confidence limits. Similar findings were made in at least 3 other experiments.

Table I

mAb	Specificity	Native Virus	Denatured Virus
RA4-44	HA2	1239	3861
RA5-22	HA2	1431	8406
RA6-30	HA2	1008	6687
RA8-57	HA2	1137	4515
H18-L10	HA2	2007	4866
H28-E23	HA1	645	15398
CM1-4	HA1	5454	6657
Y8-11A5	Host component	15510	12384
H16-L10	NP	12015	234
H19-S24	NP	3681	9042
TC-2C6	Sendai HN	162	129

The mAbs indicated were tested for binding to native vs. denatured virus in an indirect RIA in which primary Abs were incubated for 2 h at room temperature and bound Ab detected using radioiodinated rabbit anti-mouse Ig. Y8-11A5 is specific for chicken host component present on IAV and influenza B viruses that is likely to be a carbohydrate (24). TC-2C6 (25) was included as a negative control. Similar results were obtained in at least 3 additional experiments.

Table II
Denatured Escape Mutant Viruses Recapitulate Native Virus Antigenicity

Virus	PR8	JV1	JV3	JV2	KV2	AM17	BV6	BV13	ZV1
mAb									
Y8-10C2	4170	530	660	760	789	855	2943	9038	4071
H36-26	9015	11386	9638	10495	7921	8757	1802	983	8952
H36-38	3505	5819	5095	5544	3548	4187	752	767	5121
H33-23	15271	12199	14025	13714	11744	13349	13727	17203	961
H2-5A4	8321	8947	6524	9176	8184	9910	5687	13051	960
H18-L10	12186	10386	9092	9580	8545	10239	10183	13448	10931
H19-S24	16794	17514	16456	16555	17182	17590	16313	17051	17803
M2-1C6	19902	18900	18772	19561	18737	19719	19906	19477	20223

Purified antigenic mutant viruses with the amino acid substitution indicated were denatured and absorbed to polyvinyl 96 well plates. The mAbs indicated were incubated with virus for 20 h and bound antibody detected by double sandwich RIA using rabbit anti-mouse Ig followed by radioiodinated goat anti-rabbit Ig. Each mAb was tested against *wt* virus over a 100-fold range of subsaturating concentrations. Grey squares indicate mAb binding to variant virus less than its binding to *wt* virus at the 100-fold dilution. This corresponds to at least a 100-fold decrease in antibody binding affinity to the variant vs. binding to *wt* virus. Similar findings were made in 2 other experiments.

Table III

mAb-Induced Refolding Extends to other Epitopes

	Epitope	Native	Denatured
Cold mAb			
None		2189	1865
H19-S24	NP	2020	2570
H18-L10	HA2	2939	2696
Y8-10C2	Sa	244	289
Y8-1A6	Sa	619	2483
H36-26	Sb	1668	12815
H33-23	Ca	1654	7500

Polyvinyl wells containing native or denatured PR8 were incubated for 48 h with the mAbs indicated, washed, and incubated for 24 h with 4×10^6 CPM radioiodinated Y8-10C2. After washing, bound Y8-10C2 was determined by γ -counting. Similar findings were made in an additional experiment.