

Modulation of Immunodominant Sites in Influenza Hemagglutinin Compromise Antigenic Variation and Select Receptor-binding Variant Viruses

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Summary

The regions of antigenic variation in influenza hemagglutinin (HA) are located on surface-accessible regions in the three-dimensional structure of the HA1 monomer. The aim of this study was to establish whether a novel variant virus, IMUT4, in which we had mutated specific amino acid residues (HA1 63, 144, 158, and 193) in these regions, previously shown to be immunodominant for CBA/Ca mice, would either (a) establish holes in the antibody (ab) repertoire or (b) preclude further antigenic variation in IMUT4. CBA/Ca mice were able to mount a neutralizing ab response to IMUT4 infection and molecular recognition sites were established by sequencing of the HA genes of monoclonal antibody (mAb)-selected laboratory variants of wild-type X31 virus (HA1 131, 145, 155, and 196). However, each of these mAbs failed to select further antigenic variants of IMUT4, *in ovo*, but rather a receptor binding mutant (HA1 190 Glu → Asp; 226 Leu → Gln) that was still recognized by the selecting mAb, specific for HA1 155 of X31 virus. The facility for antigenic variation in influenza would appear to be compromised, therefore, by targeted mutation of immunodominant sites, as initially proposed by S. Fazekas de St. Groth (Fazekas de St. Groth, S. 1977. Antigenic, adaptive and adsorptive variants of the influenza haemagglutinin. *In* Topics in Infectious Diseases. Vol. 3. R.G. Laver, H. Bachmayer, and R. Weil, editors. Springer-Verlag, Vienna. 25–48.). It is interesting to note that recent isolates of the H3 subtype, (e.g., A/Beijing/92) obtained between 1991 and 1993, contain the same substitutions at HA1 190 and 226, which may indicate similar constraints to immune evasion and the relevance of our findings to antigenic variation in the human population.

Envelope glycoproteins of RNA viruses such as the hemagglutinin (HA) of influenza undergo frequent antigenic change as a result of immune pressure from the host neutralizing Ab response acting in concert with the error-prone virus polymerase. This presents a formidable problem to successful vaccine design. A desirable goal would be to elicit heterotypic immunity to more conserved regions of the molecule, proximal to the receptor binding site. Sequence analyses of influenza A HA genes from natural and laboratory variant viruses have identified amino acid residues that are featured in antigenic drift and thereby define the recognition sites for neutralizing Ab (1–6). In the three-dimensional structure of the bromelain-cleaved HA trimer (7, 8), antigenic drift residues are clustered in five surface accessible regions on the membrane distal ectodomain of the HA1 subunit (antigenic sites A, B, C, D, and E) adjacent to conserved residues that constitute the receptor binding site (9).

We have found that, after natural infection of CBA/Ca mice (H-2^k) with X31 virus (H3N2 subtype), neutralizing Abs recognized a limited number of antigenic sites as deduced from sequence analysis of X31 laboratory variants selected, *in ovo* with mAb (10). Such immunodominance after in-

fection of an inbred mouse strain provides an opportunity to investigate “plasticity” of the immune repertoire to antigenic variation. We have produced a novel variant virus of X31 (IMUT4) by sequential selection, *in ovo* with neutralizing mAbs representative of the previously defined Ab binding sites for CBA/Ca mice (HA1 63 Asp → Asn; HA1 144 Gly → Asp; HA1 158 Gly → Glu; and HA1 165 Asn → Ser). We demonstrate here that CBA/Ca mice are able to mount a neutralizing Ab response after infection with IMUT4 and, that therefore, there were no holes established in the immune repertoire. The molecular recognition specificity of the Ab response was determined by sequencing of the HA genes of antigenic variants of X31 virus selected *in ovo* by representative mAbs. However, these same mAbs were unable to select further antigenic variants of IMUT4 virus. After repeated rounds of selection with subneutralizing levels of a mAb specific for HA1 155 (of X31 virus), we obtained a receptor-binding variant of IMUT4 with substitutions in conserved residues that constitute part of the receptor-binding pocket, HA1 190 and 226. A limited number of mutations in immunodominant sites can therefore provide constraints to further antigenic change, be of some relevance to vaccine de-

sign, and highlight the importance of virus receptor affinity in immune evasion.

Materials and Methods

Mice. CBA/Ca mice were bred under specific pathogen-free conditions at the National Institute for Medical Research, and were infected intranasally with virus at 3–4 mo of age.

Viruses. All influenza viruses were grown in the allantoic cavity of 10-d-old embryonated eggs. The X31 virus is a recombinant between A/Aichi/2/68 and A/PR/8/34 which expresses surface glycoproteins of the H3N2 subtype and PR8 internal proteins. Laboratory-selected mutants were obtained by mixing equal volumes of allantoic fluid from X31-infected embryonated eggs and ascitic fluid containing anti-HA mAb. The virus-mAb mixture was incubated at ambient temperature for 30 min and then used to inoculate embryonated eggs. Alternatively, for IMUT4, virus was incubated with serial dilutions of mAb and, exposed to sequential selection (x3) so as to isolate an escape mutant. The variants obtained were cloned in ovo by limiting dilution. The virus was purified from ~2.5 liters of allantoic fluid by precipitation with polyethylene glycol (5%) followed by sucrose gradient (15–40% [wt/vol]) centrifugation.

Production of mAb. CBA/Ca mice were infected intranasally with 10^6 PFU (50 μ l allantoic fluid) of IMUT4 virus and boosted 8 wk later with 2×10^8 PFU administered by intraperitoneal injection. 3 d after boosting, splenic lymphocytes from individual donors were used for the production of mAb. Hybridomas were screened for anti-HA activity by hemagglutination inhibition (HI) assays using turkey erythrocytes.

Nucleotide Sequence Analyses of the HA Genes of Laboratory Mutant Viruses. RNA was phenol extracted from purified virus suspensions followed by an ether wash. Nucleotide sequences were determined using the dideoxynucleotide chain terminating method, as described elsewhere (11).

Results

Immunodominant Antigenic Sites. We have shown previously (10) that, after intranasal infection with X31 virus, the neutralizing Ab response of CBA/Ca mice is focused on a few immunodominant regions of the HA1 subunit. This was deduced from sequence analyses of laboratory variant viruses selected with mAbs that had been established from a panel of individual donor mice. The frequency and specificity of CBA/Ca-derived mAbs are summarized in Table 1, showing that the major neutralizing epitopes are centered on HA1 158 Gly \rightarrow Glu (60%) and HA1 63 Asp \rightarrow Asn (17%). Several novel laboratory mutants were also selected with single amino acid substitutions that had not been reported previously for H3 subtype viruses (HA1 62 Ile \rightarrow Arg; HA1 165 Asn \rightarrow Ser; and HA1 273 Pro \rightarrow Leu).

Sequential Selection of Variant Virus, IMUT4. We wished to determine whether each of the antigenic sites that had been featured in the CBA/Ca repertoire (Table 1) could be mutated (by mAb selection) so as to produce a laboratory variant that was no longer recognized by this particular mouse strain. The strategy for sequential Ab selection, in ovo, is summarized in Fig. 1. It should be emphasized that it was essential, at each selection step, to establish that antigenically

Table 1. Frequency and Specificity of Neutralizing mAb established from Individual CBA/Ca Donors

Antigenic specificity		Frequency
HA1 62	Ile \rightarrow Arg*	1/40 [†]
HA1 63	Asp \rightarrow Asn	7/40
HA1 129	Gly \rightarrow Trp	2/40
HA 144	Gly \rightarrow Asp	2/40
HA1 158	Gly \rightarrow Glu	24/40
HA1 165	Asn \rightarrow Ser	1/40
HA1 188	Asn \rightarrow Asp	2/40
HA1 273	Pro \rightarrow Leu	1/40

* Antigenic specificity was deduced from the single amino acid substitutions in laboratory mutants of X31 virus, selected in ovo with the corresponding mAb.

[†] A total of 40 mAbs were established from five CBA/Ca donors after intranasal infection with X31 virus.

distinct regions of HA1 were not perturbed so as to preclude further mAb selection. After each mAb treatment, escape mutants were cloned, their HA genes sequenced, and their reactivity for unrelated mAb specificities determined by HI assays.

IMUT2: HA1 158 Gly \rightarrow Glu; 63 Asp \rightarrow Asn. The starting point for selection was a laboratory variant of X31 virus, HA1 158 Gly \rightarrow Glu, since this was the most antigenic and frequently selected escape mutant. After further mAb selection, we confirmed, by cDNA sequencing, that IMUT-2 contained the relevant substitution HA1 63 Asp \rightarrow Asn. This introduced an additional N-glycosylation site (Asn₆₃ Cys₆₄ Thr₆₅) and the presence of an oligosaccharide side chain abrogated recognition by mAbs specific for HA1 62 (antigenic site C) or HA1 273 (antigenic site E), as shown in HI assays (data not shown). Therefore, these mAbs were not used for any further selection.

IMUT3: HA1 158 Gly \rightarrow Glu; 63 Asp \rightarrow Asn; 144 Gly \rightarrow Asp. A mAb specific for HA1 144 (Gly \rightarrow Asp) was shown to recognize IMUT2 in HI assays and was able to select an escape mutant with the corresponding substitution.

IMUT4: HA1 158 Gly \rightarrow Glu; 63 Asp \rightarrow Asn; 144 Gly \rightarrow Asp; 193 Ser \rightarrow Arg. A mAb specific for HA1 165 (Asn \rightarrow Ser) had previously selected (10) a laboratory mutant of X31 virus with the loss of an N-glycosylation site (Asn₁₆₅ Val₁₆₆ Thr₁₆₇). This same Ab now selected a variant of IMUT3 with an alternative substitution, HA1 193 Ser \rightarrow Arg.

Neutralizing mAb Elicited by IMUT4 Infection. We wished to determine whether CBA/Ca mice were able to mount a protective immune response to infectious intranasal challenge with IMUT4. Despite the pronounced virulence ($LD_{50} < 5 \times 10^6$ PFU) of both wild-type X31 and IMUT4 viruses, mAbs were established from two CBA/Ca donors that received a minimal infectious challenge with IMUT4 (10^6 PFU). A predominance of the IgG2a isotype was consistent

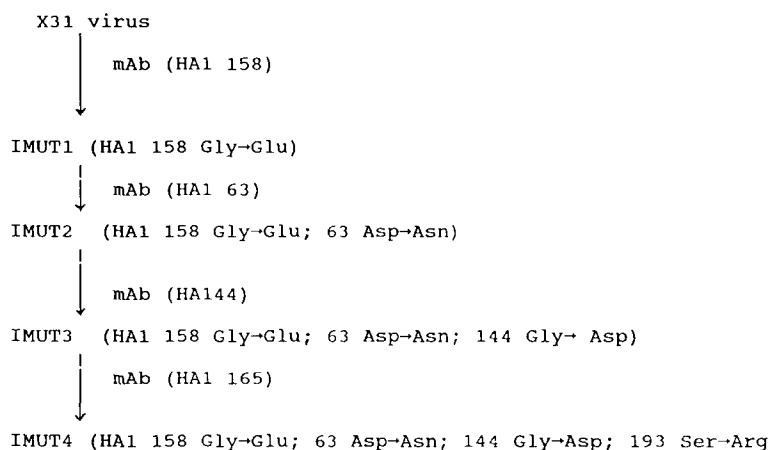


Figure 1. Sequential mAb selection of laboratory variants of X13 virus, indicating the specificity of the selecting antibody and mutations in the corresponding laboratory variants.

with recruitment of a Th1 type T cell response to virus infection.

Nucleotide Sequence Analysis of Mutant HAs. The neutralizing mAbs generated from IMUT4-infected mice were used to select variants of the parental X31 virus in the first instance.

A definitive assignment of antigenic specificities was possible for representative mAbs from donors F₁ and F₃ by sequencing of laboratory variants. Table 2 summarizes the single amino acid substitutions in X31 variant viruses VFX-11 (HA1 196 Val → Ile) VFX-13 (HA1 131 Thr → Ile), VFX-14 (HA1 145 Ser → Asn), VFX-32 (HA1 196 Val → Ile), and VFX-33 (HA1 155 Thr → Ile). It should be emphasized that between 15 and 20 mAbs were established from each donor and were representative of these specificities in binding assays.

It is of interest that (apart from HA1 145) these changes have not been reported previously for laboratory variants of the H3 subtype. And although the HA1 155 Thr → Ile change has occurred in recent H3 natural isolates (see below), HA1 196 and HA1 131 are conserved residues.

Table 2. Amino Acid Substitutions in Laboratory Variants of X31 Virus, Selected with mAbs from Individual CBA/Ca Donors after Infection with IMUT4

mAb	Isotype	Laboratory variant	Amino acid change (HA1)
FO1-11	IgG2a	VFX-11	196 Val → Ile*
FO1-13	IgG2a	VFX-13	131 Thr → Ile
FO1-14	IgG2a	VFX-14	145 Ser → Asn
FO3-32	IgG2a	VFX-32	196 Val → Ile
FO3-33	IgG2a	VFX-33	155 Thr → Ile

* Single amino acid substitutions in HA1 deduced by sequencing HA genes of laboratory variants.

We failed, however, to obtain variants of the immunizing IMUT4 virus using the same mAbs and a variety of selection procedures, including single round selection in cultured MDCK cells. After repeated in ovo selection (three rounds at subneutralizing levels) with mAb FO3-33 (specific for HA1 155 of X31 virus) a resistant mutant IMUT4-V33 was obtained that differed from IMUT4 by two further changes: HA1 190 Glu → Asp, and 226 Leu → Gln (Table 3).

IMUT4-V33 Is a Receptor-binding Mutant. Glu 190 and Leu 226 are conserved residues in H3 subtype viruses and are implicated in receptor binding, (9, 12, 13). Since we had screened the mutant viruses by HI assay, this would also detect viruses with increased or altered receptor affinity. Therefore, we examined the recognition specificity of mAb VFX-33 (HA1 155) for X31 or IMUT4-V33 by ELISA and found that it reacted equally to wild type and mutant virus (data not shown). IMUT4-V33 is therefore not an antigenic variant.

Changes in Receptor-binding Specificity of Mutant HAs. The drift substitution, HA1 155 Thr → Tyr, in H3 subtype iso-

Table 3. Amino Acid Substitutions in a Laboratory Variant of IMUT4 Virus, Selected with mAb Specific for HA1 155 (Thr → Ile), FO3-33

mAb	Laboratory variant	Amino acid change (HA1)
FO3-33*	IMUT4-V33	190 Glu → Asp 226 Leu → Gln 63 Asp → Asn† 144 Gly → Asp† 158 Gly → Glu† 193 Ser → Arg†

* The specificity of the selecting mAb for HA1 155 (Thr → Ile) was determined by sequencing the HA gene of a laboratory variant of X31 virus (see Table 2).

† Amino acid substitutions present in the "parental" IMUT4 virus.

Table 4. Changes in Receptor-binding Specificity of Laboratory Variants as Determined by Hemagglutination of Horse Erythrocytes, and Inhibition of Hemagglutination by Horse Serum

Virus	HAU	Horse RBC	HI
	Turkey RBC		Turkey RBC; Horse serum
X31	1,204	<2	10,240*
VFX-33	1,024	1,024	10,240
IMUT4-V33	1,024	<2	<20
X31-HS	1,024	<2	<20

* The titer for hemagglutinin inhibition by serial dilution of horse serum.

lates has been implicated in conferring agglutinin activity for RBC that contain *N*-glycolyl neuraminic acid residues (such as horse RBC) whereas A/Aichi/2/68 exhibits strict specificity for *N*-acetyl neuraminic acid (14). It was of interest, therefore, to determine the agglutinin specificity of VFX-33 (HA1 155 Thr → Ile). Table 4 shows that, in contrast to wild-type X31 virus, VFX-33 agglutinates turkey and horse RBC equally well, illustrating the importance of HA1 155 in receptor-binding specificity.

The HA receptor of human H3 isolates has specificity for the anomeric linkage of sialic acid and preferentially agglutinates RBC containing sialyl α 2,6 galactose residues. A receptor-binding variant X31-HS (HA1 226 Leu → Gln) had been selected previously for its resistance to hemagglutinin inhibition by horse serum α ₂-macroglobulin, and had increased affinity for the sialyl α 2,3 anomeric linkage (12). In Table 4 we have compared the susceptibility of X31, X31-HS, and IMUT4-V33 to hemagglutinin inhibition by horse serum. Both mutant viruses have a common substitution (HA1 226 Leu → Gln), and are equally resistant to horse serum inhibition, thereby confirming their altered receptor-binding specificity for sialic acid.

Discussion

This study has demonstrated that the introduction of key mutations in the globular head region of the HA1 subunit provides structural constraints on further antigenic change in influenza under immune pressure of neutralizing mAb. Even so, such constraints can be countered (by the virus) by changes in virus receptor specificity (or affinity), thereby providing an additional mechanism of immune evasion.

Our initial aim was to establish whether introduction of predetermined mutations in antigenic regions of the HA molecule, previously shown to be immunodominant in the neutralizing Ab response of CBA/Ca mice to X31 virus infection, would either establish holes in the repertoire, or conversely, produce a variant virus that was no longer able to mutate (in vitro) under selective pressure of neutralizing mAb. To this end, we produced a novel laboratory variant of X31 (IMUT4: HA1 63 Asp → Asn; 144 Gly → Asp; 158 Gly

→ Glu; and 193 Ser → Arg) by sequential antibody selection, in ovo. After infectious challenge with IMUT4, it was possible to obtain neutralizing mAbs from CBA/Ca mice and their recognition specificity determined by the selection of laboratory variants and sequencing of their HA genes. Therefore, there was no hole in the host-protective Ab repertoire.

All of the mAbs recognized the wild-type X31 virus and selected variants with single substitutions at HA1 131 Val → Ile, HA1 155 Thr → Ile, HA1 196 Val → Ile, or HA1 145 Ser → Asn. Most of these are novel changes that have not been reported previously for laboratory variants and include changes at conserved positions (Val 131 or Val 196) that have featured in antigenic drift. Despite repeated attempts, we failed to select laboratory variants of IMUT4 using the same mAbs and selection protocol. Finally, after three selection cycles with mAb FO3-33 (specific for HA1 155), a laboratory variant of IMUT4 was obtained with two further amino acid substitutions at HA1 190 Glu → Asp, 226 Leu → Gln. These are conserved residues that form an integral part of the receptor-binding pocket (9, and Fig. 2).

Sequence analyses of natural variants of the H3 subtype isolated in the past 20 yr indicate that more than 35 drift substitutions have occurred within antigenically significant regions of HA1. (3, 8). It is surprising, therefore, that the introduction of such a limited number of substitutions in IMUT4 impose such constraints on further antigenic change. Although IMUT4 elicits neutralizing Ab responses after natural infection, and specificity is readily determined by mAb selection of X31 variant viruses, we have failed to select further antigenic variants of IMUT4.

Given the extensive correlates that are available for antigenic variability, receptor-binding specificity, and three-dimensional structure of the HA of X31 virus, we are able to interpret our findings at the molecular level. In the three-dimensional structure of HA complexed with sialyl lactose (9), the receptor-binding site is a cleft at the membrane distal tip surrounded by variable residues that have featured in antigenic drift. Fig. 2 is a schematic representation of the receptor-binding site occupied by sialic acid (as depicted in reference 9), and indicates its possible hydrogen-bonded contacts to

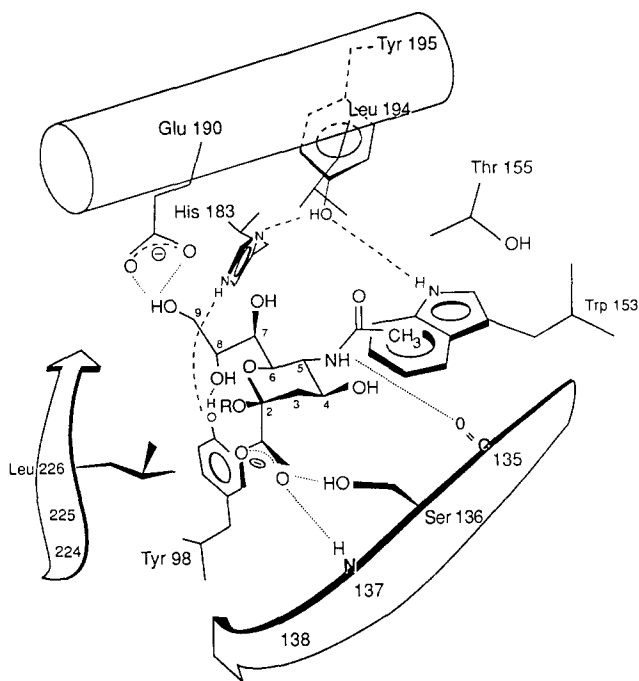


Figure 2. A schematic model of the HA receptor binding site indicating hydrogen bonding of bound sialic acid. (9).

the receptor. The floor is made of Tyr 98 and Trp 153, and Glu 190, Leu 194 project down from a short α -helix which, together with His 183 and Thr 155, constitute the rear of the cleft. Residues 134-138 are on the right and 224-228 are on the left. Consider below the residue changes in mutant HAs that we have identified in this study.

IMUT4-V33: 190 Glu \rightarrow Asp, 226 Leu \rightarrow Gln. In H3 subtype viruses, 190 Glu is a conserved negatively charged residue projecting down into the cleft and hydrogen bonded to sialic acid. The topographic relation of Glu 190 to Leu 226, and their role in receptor function is evident from their molecular location. No laboratory variants have been reported for Glu 190 or Leu 226 but, it is interesting to note that recent natural isolates of the H3 subtype have the same substitutions, 190 Glu \rightarrow Asp and 226 Leu \rightarrow Gln (Dr. A. Hay, World Influenza Centre, National Institute for Medical Research, personal communication). This highlights the structural constraints imposed on mutations within the conserved receptor-binding site, and the possible significance of such

changes in the selection of natural variant viruses with altered receptor specificity.

A receptor-binding variant of X31, 226 Leu \rightarrow Gln, selected in the presence of horse α_2 -macroglobulin, differs from wild-type virus in its ability to agglutinate RBC containing sialyl $\alpha_{2,3}$ linkages (12). The crystal structure of the variant HA (9) indicates that minimal changes have occurred in the receptor pocket so as to accommodate the additional hydrogen-bonding potential of Gln 226.

VFX-33: 155 Thr \rightarrow Ile. In the crystal structure the acetamidomethyl group of sialic acid is facing residue 155, and the drift substitution 155 Thr \rightarrow Tyr in natural isolates confers increased affinity for *N*-glycolyl sialic acid residues (14). Mutant VFX-33 also exhibits specificity for *N*-glycolyl sialic acid and agglutinates horse RBC, and it is interesting to note that the same 155 Thr \rightarrow Ile change has occurred in natural isolates of the H3N2 subtype (A/CAEN/83 or A/SOF/84; Dr. J. J. Skehel, personal communication).

There are previous reports of influenza variant viruses with altered receptor-binding specificity that had been selected, *in vitro* with subneutralizing amounts of either polyclonal Ab (15) or a mixture of mAbs specific for several distinct antigenic sites (16). Moreover, mAb-selected antigenic variants can exhibit different specificities for enzymatically or chemically altered RBC (17). Daniels et al. (13) have described a mAb to X31 virus that failed to discriminate between natural isolates of the H3 subtype and selected receptor-binding variants of X31 with substitutions at HA1 193 Ser \rightarrow Asn, 218 Gly \rightarrow Glu, 226 Leu \rightarrow Pro, or deletion of 224-230. With the exception of HA1 193, these changes do not abrogate Ab recognition of mutant virus (in ELISA assays) and therefore qualify as receptor-binding mutants.

A significant finding of this study is that constraints can be imposed on antigenic variation in influenza hemagglutinin, by a limited number of mutations in defined Ab recognition sites, resulting in the selection of a receptor-binding variant virus (HA1 190 Glu \rightarrow Asp, 226 Leu \rightarrow Gln). This had been the initial aim of Fazekas de St. Groth (15), some 20 yr ago, namely to anticipate antigenic drift in influenza, by Ab selection, so as to produce a candidate vaccine strain. Since these same residue changes have been found in recent human H3 isolates, this may indicate that there are similar constraints to further antigenic change within the human population. Our findings emphasize that the receptor-binding specificity (or affinity) of virus may be an important factor in immune evasion of neutralizing Ab.

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