Structural Assignment of Novel and Immunodominant Antigenic Sites in the Neutralizing Antibody Response of CBA/Ca Mice to Influenza Hemagglutinin

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Summary

Information on the antigenic structure of influenza hemagglutinin (HA) has been deduced previously from sequence analyses of laboratory mutant viruses selected, in vitro, with neutralizing monoclonal antibody (mAb) established exclusively from BALB/c (H-2^d) mice; and there has been no attempt to investigate the influence of host genetic background, or natural route of infection, on the protective antibody repertoire. CBA/Ca mice are extremely sensitive to X31 virus infection, and in the present study a structural analysis was made of the antibody repertoire, by direct sequencing of the HA genes of laboratory mutant viruses selected, in ovo with mAb from CBA/Ca mice primed by natural infection with X31 virus at two different infectious doses. Single nucleotide substitutions in the HA genes of mutant viruses identified both novel and immunodominant antigenic sites on the HA1 subunit: a majority of mAbs, from different donors, were of the IgG2a isotype and were specific for HA1 158 Gly. In addition, novel laboratory mutants were obtained containing substitutions in the HA1 subunit that had not been reported previously for H3 subtype viruses, either natural variants or laboratory mutants, at residues: HA1 62 Ile → Arg; HA1 165 Asn → Ser (resulting in the loss of a *N*-glycosylation site); and HA1 273 Pro → Leu. Our findings suggest that host genetic background and/or a natural route of infection may be significant factors in the selection of different and distinct neutralizing antibody responses to influenza HA and therefore be of some relevance in our further understanding of the immune pressure for antigenic drift, and the immunogenic features of a protective antigen.

A definitive assignment of antibody recognition sites, or B cell epitopes of foreign proteins, requires information on the three-dimensional structure, obtained from crystallo-graphic data for antigen-antibody complexes, whereas, in most instances, B cell epitopes have been identified by indirect means such as antibody reactivity with families of phylogenetically related proteins (e.g., myoglobin or cytochrome *c*) (1), or by protein modification using either chemical procedures or site directed mutagenesis (2). For infectious pathogens that exhibit antigenic variation such as the envelope glycoproteins of RNA viruses, a structural analysis of mAb-selected escape mutants provides a convenient approach for identifying critical residues necessary for antibody recognition. A primary example of this approach has been the extensive studies undertaken with influenza haemagglutinin (HA)[1] (3–7). Sequence analyses of the RNA genes of mAb-selected laboratory mutants have identified single amino acid substitutions corresponding to surface regions on the membrane distal domain of the HA1 subunit that have also featured in antigenic drift (6). Analysis of laboratory mutant viruses therefore provides a powerful tool for correlating the immunogenic features of a protective antigen with three-dimensional structure, and for studies on the fine specificity and diversity of antibody recognition.

We have found that CBA/Ca mice, in contrast to the majority of inbred and congenic strains, are highly susceptible to infection with X31 virus (H3N2 subtype; our unpublished observations) and we wished to determine whether there was a correlation between disease susceptibility and the neutralizing antibody repertoire to HA. We have found that a majority of laboratory mutant viruses, selected with a panel of mAbs from different donors, differ from the immunizing X31 virus by the same single amino acid substitution, HA1 158 Gly → Glu. Additional mutant viruses were characterized that contained novel substitutions at HA1 62 (Ile → Arg), or HA1 165 (Asn → Ser; resulting in the loss of a *N*-glycosylation site) or HA1 273 (Pro → Leu). These substitutions have not been reported previously for either natural variants or mAb (BALB/c)-selected mutant viruses of

[1] *Abbreviations used in this paper:* HA, hemagglutinin; HI, hemagglutination inhibition.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/04/0953/07 $2.00
Volume 173 April 1991 953–959

the H3 subtype. There may, therefore, be two important considerations in an analysis of the antibody repertoire for a protective antigen: first, a natural route of infection may elicit a long-lived B memory cell response that is restricted to fewer and possibly different antigenic sites than obtained after immunization; and second, that the host genetic background may be a significant determinant in the specificity of the neutralizing antibody response.

Materials and Methods

Viruses. All influenza viruses were grown in the allantoic fluid 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 (8). 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 left at room temperature for 30 min and then used to inoculate embryonated eggs. The variants obtained were cloned, in ovo, by limiting dilution and subsequently grown in large scale using on average 300 eggs per virus. The virus was purified from ~2.5l of allantoic fluid by precipitation with polyethylene glycol (5%) followed by sucrose gradient (15–40% [wt/vol]) centrifugation.

Mice. CBA/Ca mice were bred under specific pathogen-free conditions at the National Institute for Medical Research (NIMR). Mice were infected at 3–4 mo of age.

Production of mAb. CBA/Ca mice were infected intranasally with either 1.5×10^7 PFU (high dose) or 2×10^6 PFU (low dose) of X31 and boosted 6 wk later with 2×10^8 PFU administered by intraperitoneal injection. 3 d after boosting, splenic lymphocytes from individual donors were fused with P3-X63-Ag8653 myeloma cells (9) after the fusion protocol and culture conditions described by Fazekas de St. Groth and Scheidegger (10). Hybridomas were screened for anti-HA activity by hemagglutination inhibition (HI) assay.

Hemagglutination Inhibition (HI) Assay. mAb containing tissue culture medium (50 μl) was mixed with X31 virus (8HAU; 25 μl) and incubated at room temperature for 30 min. 25 μl of a 2% turkey RBC suspension was added and incubated for a further 30 min at room temperature to allow agglutination.

mAb Isotypes. The isotypes of each mAb used in this study were determined by ELISA; mAb bound to the X31 virus was detected using biotinylated anti-mouse Ig isotype-specific antibodies and a streptavidin-alkaline phosphatase conjugate.

Nucleotide Sequence Analyses of the HA Genes of Laboratory Mutants of X31. RNA was extracted from purified virus suspensions (2–10 mg/ml). RNA was phenol extracted followed by ether wash. Sequences were determined using the dideoxynucleotide chain-terminating method (11) and the procedure used has been described elsewhere (12). Briefly, ~6 μM of ^{32}P-labeled primer was mixed with 1 μg RNA and the primer extension reaction was carried out using individual dNTP at a final concentration of 0.4 mM and ddNTP at a concentration of 0.1 mM in a total volume of 3 μl. The primers (kindly provided by Dr. R.S. Daniels, NIMR) were as follows: 6-AAGCAGGGGA-15; 191-TGCTACTGAGCT-202; 367-GTTACCCTTATG-388; 628-ACCCGAGCACG-638; 778-GGACAATAGT-787, and numbered according to the sequence of X31 hemagglutinin cDNA (13).

Results

We have found that CBA/Ca mice are highly susceptible to intranasal infection with X31 virus ($LD_{50} < 5 \times 10^6$ PFU) as compared with most other inbred and congenic strains of mice (e.g., BALB/c, BALB.K, BALB.B, or B10 congenics; $LD_{50} > 2 \times 10^7$ PFU; our unpublished results). This is illustrated in Fig. 1 where the weight loss (and death) after infection of CBA/Ca and BALB/c mice has been compared. Since BALB/c mice do not express the autosomal dominant MX1 gene that confers resistance to influenza (14), the differences in susceptibility to influenza X31 infection between these two inbred strains of mice must therefore be under the control of other factors. The purpose of this study was to determine the neutralizing antibody epitopes of CBA/Ca mice after natural infection with X31 virus.

HA-specific mAb

A panel of 40 anti-HA neutralizing mAbs was derived from five CBA/Ca mice infected with X31 virus at two different infectious doses, either 2×10^6 or 1.5×10^7 PFU, and are

Table 1. *Immunoglobulin Isotypes of Anti-HA mAb*

JCB-2		JCB-3		JCB-4		LD4		LD6	
Clone	Isotype	Clone	Isotype	Clone	Isotype	Clone	Isotype	Clone	Isotype
2B-41.1	IgG2a	3B-1.1	IgG2a	4B-40.7	IgG3	4C4	IgG2a	B72	IgG2a
-7.3.2	IgM	-8.1	IgG2a	-35.4	IgG2a	5D2	IgG2a	C98	IgG2a
-29.1	IgG2a	-4.3	IgG2b	-2.1	IgG2a	6G3	IgG2a	A76	IGg2b
-37.1	IgA	-3.6.2	IgG2a	-3.1	IgG2a	9G12	IgG2a	A122	IgG2b
-5.1.1	IgG2a	-3.6.3	IigG2a	-4.3	IgG1	8D11	IgG2a	D91	IgG2a
-6.1	IgG2a	-5.1	IgG2b	-20.1	IgG2a	3F9	IgG2a	D101	IgG2b
-21.3	IgM	-7.1	IgG2a	-12.1	IgG1	1A12	IgG2a	G63	IgG2a
		-8.2	IgG2a	-29.1	IgG2a	7G11	IgG2a	B28	IgG2b
		-6.1	IgG2a					D92	IgG2a

Donors JCB-2, -3, -4 (high dose infection, 1.5×10^7 PFU); Donor LD4, LD6 (low dose infection, 2×10^6 PFU).

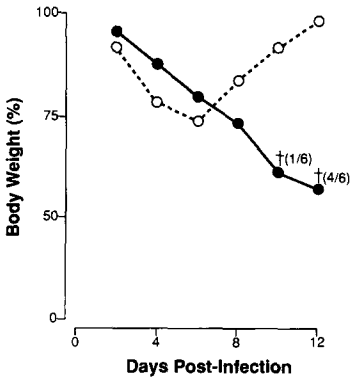

Figure 1. A comparison of the virulence of X31 virus in CBA/Ca and BALB/c mice as determined by body weight and survival after intranasal infection with 10^7 PFU. (○) BALB/c mice; (●) CBA/Ca mice + mortality.

listed in Table 1. The predominant Ig isotype among these mAb was IgG2a. In an initial attempt to determine the level of diversity of the secondary antibody response to HA, the mAbs were tested by HI assay against a panel of laboratory mutants having known single amino acid substitutions within the HA1 subunit (Fig. 2). This panel of mutant viruses allowed us to examine the effects on antibody binding of mutations within four of the five antigenic sites on the HA1 subunit as defined by Wiley et al. (6). The results indicated a high degree of diversity among the mAbs tested. For instance, out of 24 mAbs generated from donors JCB-2, -3, and -4, 15 different fine specificities were seen with respect to their HI activity. However, it was not possible to define precisely which amino acid residues in the HA1 subunit were being recognized by individual antibodies. Such extensive diversity was consistent with previous reports for the BALB/c repertoire (15).

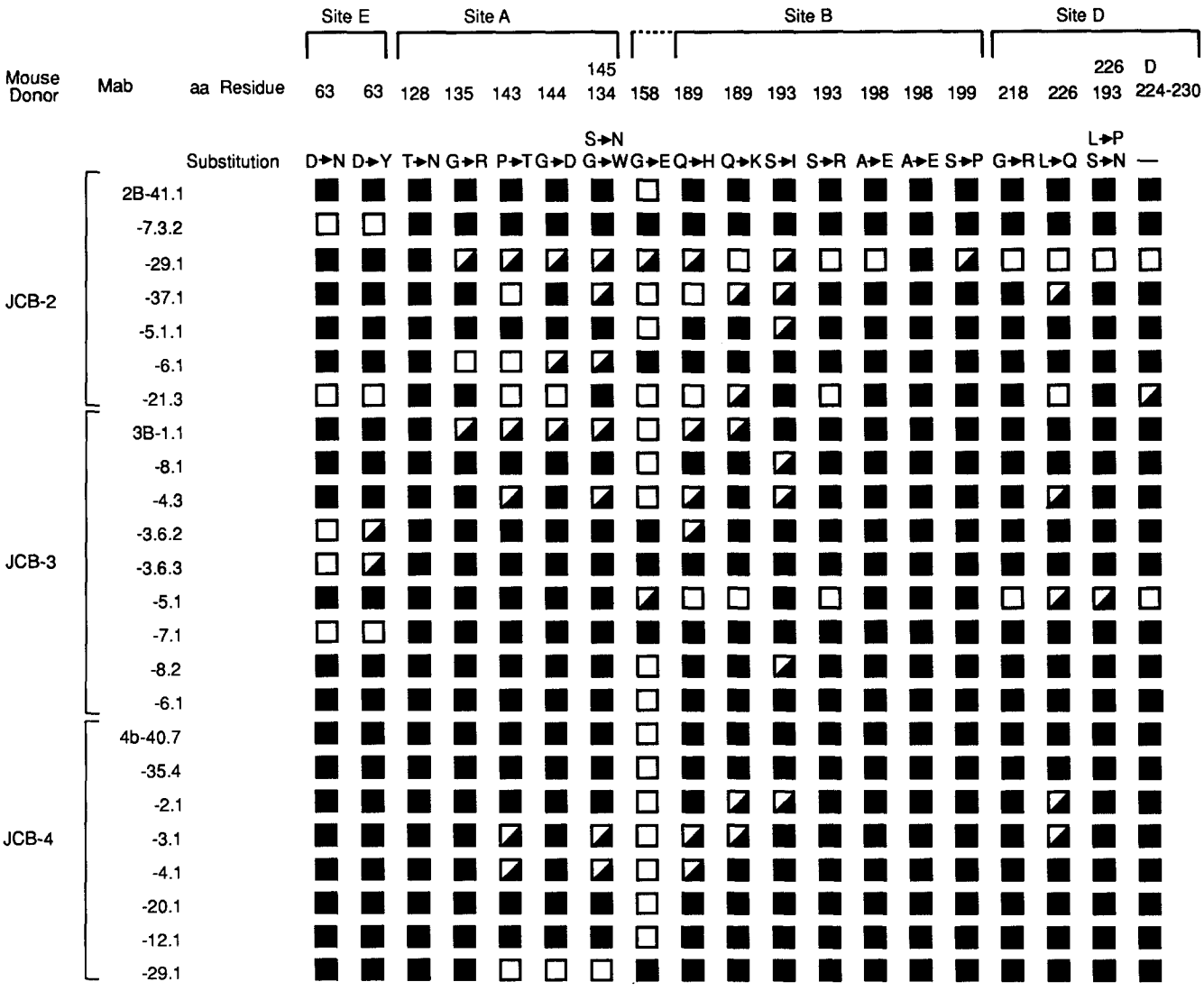

Figure 2. A summary representation showing the complex pattern of HI reactivity of anti-HA mAb against a panel of laboratory mutant viruses containing single amino acid substitutions within the HA1 subunit and a deletion mutant, residues 224–230. (■) Inhibition of hemagglutination; (◪) partial inhibition of hemagglutination; (□) no inhibition of hemagglutination. All HI assays were performed using viruses at a concentration of 8HAU/25 μl, using two or more dilutions of neutralizing mAbs and in direct comparison of reactivity of the mAb for wild type X31 and mutant viruses.

Sequence Analysis of mAb-selected Laboratory Mutants

To obtain a definitive assignment of the neutralizing antibody binding sites of HA1, recognized by CBA/Ca mice, each of the above mAbs was used to select a laboratory mutant of X31 virus. The HA gene of each cloned laboratory mutant virus was sequenced and the amino acid substitution that had occurred was deduced from the nucleotide sequence data. Our findings are summarized in Table 2 and illustrate novel substitutions that have, hitherto, not been reported for either laboratory mutants or natural isolates of the H3 subtype.

HA1 158 (Gly → Glu). Residue 158 occupies a position near the tip of the HA monomer adjacent to the receptor binding site (6) (Fig. 3). A significant finding was that the majority of the laboratory variants differed from the wild type X31 virus by a single substitution at this position. Comparison of mAbs obtained from donors infected with a low or high dose of X31 virus showed that, in both groups, a majority of mAbs recognized HA1 158. This restriction of the responses was particularly striking in donor JCB-4 where seven out of eight mAbs, of differing isotypes, recognized HA1 158.

HA1 63 (Asp → Asn). This was the next most frequently selected laboratory mutant. HA1 63 lies within antigenic site E of the HA1 subunit (Fig. 3). This substitution introduces an *N*-glycosylation site (Asn_{63} Cys_{64} Thr_{65}) that alters the antigenic structure of this region and abrogates both antibody recognition (16) and antigen presentation of an I-A^d-restricted T cell epitope (17).

HA1 62 (Ile → Arg). This residue is also within antigenic site E and the HA1 62 Ile → Arg change has not been previously reported for either laboratory mutants or natural variant viruses of the H3 subtype. However, this same substitution has been reported for the HAs of avian H3 isolates.

HA1 165 (Asn → Ser). This residue change results in the loss of *N*-glycosylation site (Asn_{165}, Val_{166}, Thr_{167}) and has not been reported previously for either laboratory mutants or natural variants of the H3 subtype.

HA 273 (Pro → Leu). This residue change, occurring in antigenic site C, has not been reported for mAb (BALB/c)-selected laboratory mutants although natural variants of the

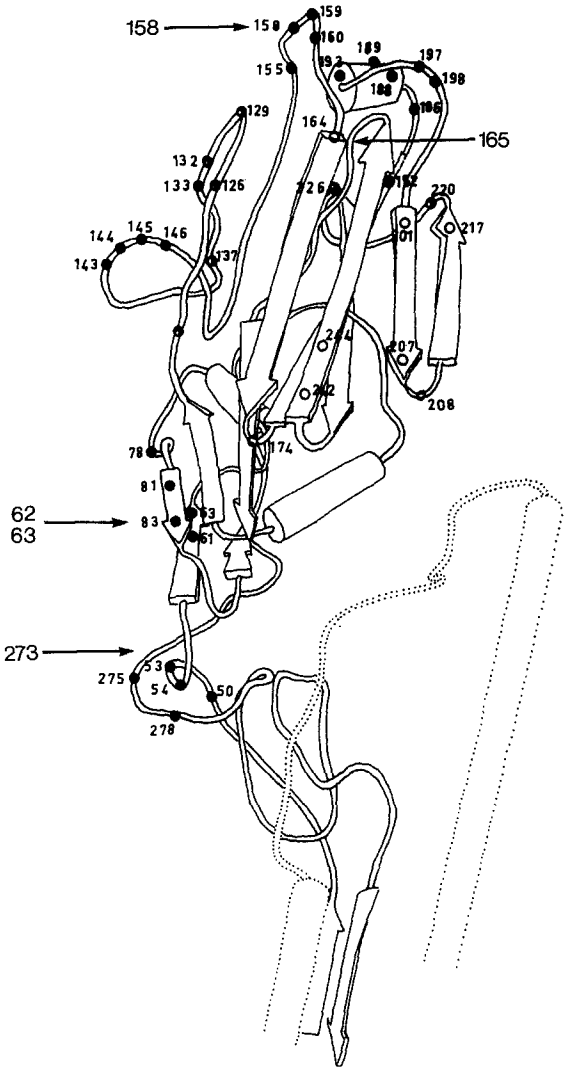

Figure 3. Schematic diagram of the three-dimensional structure of the HA1 monomer indicating residues recognized by CBA/Ca mAb (*arrows*) and the residues that have featured in antigenic drift of H3 subtype viruses (●).

Table 2. *The Amino Acid Substitutions (and Frequency) in Laboratory Variants Selected, In Ovo, by mAbs Derived from Individual CBA/Ca Donors*

Donor mice	62* I→R‡	63 D→N	129 G→W	144 G→D	158 G→E	165 N→S	188 N→D	273 P→L
JCB-2		1		1	4			1
JCB-3	1	2			5	1		
JCB-4				1	7			
LD4		2	2		2		1	
LD6		2			6		1	

* HA1 amino acid position.
‡ Laboratory mutant virus.

H3 subtype such as A/TEX/77 or A/BK/79 contain substitutions within this region at HA1 275 (Asp → Gly) and HA1 278 (Ile → Ser).

HA 129 (Gly → Glu). This is a radical change that has featured in natural variants of the H3 subtype (A/QU/70 and A/HK/71) and has a profound effect on antibody binding to the membrane distal region of HA1, as illustrated by the failure of all mAbs specific for HA1 158 to recognize laboratory mutants containing this single substitution.

The other laboratory mutants obtained in this study contained single substitutions at positions HA1 144 Gly → Asp and HA1 188 Asn → Asp within antigenic sites A and B (6), represented a minor component of the CBA/Ca repertoire, and have been reported previously for either laboratory mutants or natural variant viruses.

HA1 158 Gly → Glu Abrogates Antibody Binding

Since the HA1 158 Gly → Glu change has an effect on receptor binding (see below), it was necessary to establish that laboratory mutants containing this substitution were indeed true antigenic variants. They had been screened for in HI assays which would also detect viruses with altered receptor affinity. Therefore, each mAb was tested by ELISA assay for its ability to bind to the mutant virus which it selected. As shown in Table 3, all the mutant viruses were antigenic variants as they all showed greatly reduced ability to bind the selecting mAb as compared to X31 virus.

Table 3. *Reactivity of mAb, Specific for HA1 158, with X31 Virus and the Corresponding Laboratory Mutant Viruses HA1 158 Gly → Glu in ELISA Assays*

	Antibody titre*	
mAb	X31	X31 (158 Gly→Glu)
2B -5.1.1	$>10^5$	$<10^1$
-29.1	$>10^5$	$<10^1$
-31.1	$>10^5$	$<10^1$
3B - 6.1	10^5	$<10^1$
- 8.2	10^2‡	–
- 4.3	$>10^5$	$<10^1$
- 8.1	$>10^5$	$<10^1$
- 1.1	$>10^5$	$<10^1$
4B -12.1	10^2‡	–
-20.1	10^3‡	–
- 3.1	10^5	$<10^1$
-40.7	10^4	$<10^1$
-35.4	10^4	$<10^1$
- 2.1	$>10^5$	$<10^1$

* Titre of mAb (from ascites fluid) with X31 and with mutant virus selected by the corresponding mAb.
‡ Titre of mAb-containing tissue culture supernatant.

Discussion

Structural analyses of laboratory mutants of influenza, selected in vitro with neutralizing mAb to HA, have played a significant role in delineating the antigenically important regions of the molecule, and for evaluating the antigenic significance of amino acid substitutions in natural variant viruses (3–7). Laboratory mutant viruses differ by one, or occasionally two, amino acid substitutions in the membrane distal regions of the HA1 subunit, and these same substitutions have been identified in natural variants, thereby confirming their importance in antigenic drift. In all reported studies, neutralizing mAb to HA have been obtained from BALB/c mice, after immunization with whole virus, and shown to be specific for one of the five major antigenic sites of HA1. There have been no reports, however, of investigations of the antibody repertoire of other mouse strains (or haplotypes) or the specificity of secondary responses after natural infection. In this study, an extensive analysis was made of the neutralizing antibody repertoire of CBA/Ca mice, a strain that is highly susceptible to X31 infection. Sequence analyses of the HA genes of laboratory mutant viruses of X31 provided a structural assignment of both novel and immunodominant antigenic sites on the HA1 subunit. Our findings are summarized schematically on the three-dimensional structure of the HA monomer (Fig. 3).

Protection against influenza infection, in vivo, correlates with serum levels of neutralizing IgG antibodies directed against the membrane distal region of the HA1 subunit (18, 19). HI activity, the assay of choice herein for the screening of mAb correlates with neutralizing antibody activity and therefore provides a relevant index of the protective antibody repertoire of CBA/Ca mice. Our results have shown that the secondary antibody response is directed predominantly at HA1 158 Gly and is mainly of the IgG2a subclass. In addition, we have described novel substitutions not previously reported for laboratory mutants or natural variant viruses of the H3 subtype. The predominance of the IgG2a isotype in secondary antibody response is a characteristic trait of most viral infections in the mouse (20–23) and may illustrate the influence of antigen-specific $CD4^+$ T cells. $CD4^+$ cells have been designated as Th1 or Th2 on the basis of their lymphokine secretion profile (24): Th1 cells secrete IFN, which enhances IgG2a synthesis in vitro (25, 26), and has been shown to preferentially induce IgG2a production (27). The IgG2a isotype does not predominate in the antibody response to immunization with inactivated virus (23) thus emphasizing the role of natural infection in the selective activation of Th1 cells.

HA1 158 Gly, the major antigenic site for CBA/Ca mice, is located at the distal end of the HA1 subunit adjacent to the receptor binding site (Fig. 3). The immunodominance of this residue did not appear to be dependent on the infectious dose of virus and was most clearly illustrated by the antibody response of donor JCB-4 (Table 2): in this instance, seven out of eight mAbs recognize HA1 158, and isotype analysis has shown that this is not an in vitro artifact, such as repetitive sampling of a common clonotype within the memory B cell population, since the mAbs represent several different isotypes (IgG1; IgG2a, IgG3).

Studies with BALB/c mAbs have shown that, after immunization with influenza, the antibody response is directed predominantly at a region of the HA1 monomer designated antigenic site B (HA1 186-200; references 8, 28). Staudt and Gerhard (15), in an extensive study on mAb-binding patterns for a panel of H1 subtype variant viruses, estimated that the minimum adult BALB/c repertoire consisted of 1,500 paratopes. They also demonstrated that individual mice expressed paratypically and idiotypically distinct repertoires. However, these significant studies did not allow a definitive structural assignment of antigenic sites since the diversity of antibody reactivity did not extend to a structural analysis of mutant HA genes. In the present study, a high degree of antibody diversity was also evident in the HI reactivity patterns for a panel of variant viruses (Fig. 2) and contrasted with the limited number of antigenic changes detected at the nucleotide level. This is well illustrated by the panel of mAbs derived from donor JCB-4: seven of the mAbs were specific for HA1 158, whereas HI analysis revealed five distinct patterns of reactivity. Our findings indicate that conclusions based on HI, or antibody-binding studies alone, may indicate a degree of paratypic complexity in antibody responses that are focused on a single amino acid residue.

The receptor binding specificities of H3 subtype influenza viruses exhibit three distinct types, based on preferential binding to either one or both of the sequences (sialyl 2 → 6 gal) or (sialyl 2 → 3 gal) that are present as terminal residues in both O-linked (29) and N-linked (30) oligosaccharides of membrane glycoproteins. Residue 158, although not located within the receptor binding site, has been implicated as having an effect on receptor binding as shown by the study of Underwood et al. (31), in which it was shown that mutant viruses with a substitution at HA1 158 had decreased affinity in receptor binding assays using periodate-modified erythrocytes. It was possible, therefore, that the laboratory mutants with the HA1 158 Gly → Glu substitution, the predominant substitution selected for by CBA/Ca mAb, were in fact receptor binding mutants rather than antigenic variants. However, as the selecting mAbs were unable to bind to the mutant viruses in ELISAs, it is clear that the HA1 158 substitution affects the antigenicity of the HA molecule. Interestingly, the work of Both et al. (32) with swine influenza virus has shown that a substitution at HA1 155 Gly → Glu (the equivalent of HA1 158 in the human H3 subtype) can alter the replicative capacity, or virulence of the virus. The HA1 155 Gly → Glu change was associated with a 1,000-fold reduction in infectivity.

Our study has also identified laboratory mutants with single substitutions in regions of the HA1 subunit that hitherto have not featured in either mAb (BALB/c)-selected laboratory mutants or natural variants of the H3 subtype. For instance, HA1 165 Asn is a *N*-glycosylation site (Asn_{165}, Val_{166}, Thr_{167}) that has been conserved in all natural variants of the H3 subtype. Viruses of the H1 subtype, in contrast, are not glycosylated at the equivalent position and do exhibit antigenic variation in this region (7), suggesting that the carbohydrate moiety at HA1 165 masks the surrounding area from immune recognition. However, the mAb-selected mutant characterized in this study contains the substitution HA1 165 Asn → Ser, resulting in a loss of the consensus sequence for *N*-glycosylation. A recent report has described a laboratory mutant with the substitution HA1 167 Thr → Asn, also resulting in the loss of *N*-glycosylation and in changed receptor binding properties (33). However, this was not an antigenic variant and had been selected with β inhibitors, which are a family of mannose-binding lectins present in bovine and murine sera. In our hands, the selecting antibody was unable to bind to the HA1 165 mutant in ELISAs, indicating that this was indeed an antigenic variant and not produced as a result of a similar selection with β inhibitors present in the ascitic fluid.

A further and novel laboratory mutant contained the HA1 273 Pro → Leu change. HA1 273 is within a loop region of the HA monomer designated as antigenic site C (Fig. 3). Although this is an antigenic region (as defined by sequence analysis of natural variants) there has only been a single report of a laboratory mutant within site C, at position HA1 53 (34). However, HA1 273 has been conserved in all natural variants so far characterized. Similarly, HA1 62 has been conserved in all H3 natural isolates and has not featured in any of the mAb (BALB/c)-selected laboratory mutants, hitherto characterized.

In conclusion, the spectrum of laboratory mutant viruses selected with mAbs from CBA/Ca mice and described herein adds a number of different sites to those reported elsewhere for laboratory mutant viruses selected with mAbs from BALB/c mice (3–7) and suggests that host genetic background and/or a natural route of infection may be significant factors in eliciting a protective antibody response to influenza.

Thanks to Dr. R. S. Daniels for the gift of the primers used in this study.

Fernando Temoltzin-Palacios is the recipient of a student fellowship from Consejo Nacional de Ciencia y Technologia, Mexico.

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Received for publication 1 November 1990.

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