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FACTORS WHICH MAY INFLUENCE THE DETECTED FREQUENCY OF ANTIGENIC VARIANTS BY MONOCLONAL ANTIBODIES AND IDIOTYPY OF CLONAL RESPONSES TO INFLUENZA VIRUS HEMAGGLUTININ

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I. ABSTRACT

FACTORS THAT MAY INFLUENCE THE FREQUENCIES OF ANTIGENIC VARIANTS DETECTED BY MONOCLONAL ANTIBODIES AND IDIOTYPY OF CLONAL RESPONSES TO INFLUENZA VIRUS HEMAGGLUTININ

by

Yung-nan C. Liu

Advisor: Jerome L. Schulman, M.D.

The studies involved in my research consisted of two aspects: (I) Factors which may influence the frequencies of antigenic variants detected by monoclonal antibodies. The frequencies of antigenic variants detected by monoclonal antibodies against the same or overlapping antigenic determinants on the hemagglutinin of influenza B/Lee/40 virus were found to vary from $10^{-5.5}$ to $<10^{-8.1}$. These differences are related to differences in the avidities of the monoclonal antibodies used in selection: namely, there is an inverse relationship between antibody avidity and detected frequency of antigenic variants. This inverse relationship was also found using a single monoclonal antibody versus wild type virus and variants. Furthermore, it was found that only the highest avidity monoclonal antibody employed, cross-reacted with B/Md/59 virus suggesting that monoclonal antibody to the same antigenic site may be specific or cross-reactive depending on antibody avidity. When monoclonal antibodies to different antigenic determinants of PR8 virus

hemagglutinin were compared with respect to avidity and frequency of variation, no clear cut relationship was evident, suggesting that frequency of variation is also influenced by factors other than antibody avidity. When monoclonal antibodies against X31 hemagglutinin were employed in the studies, several observations were made: (a) an inverse relationship between antibody avidity and detected frequencies of antigenic variants was found using a single monoclonal antibody (X146) and different viruses, (b) the concentration of antibody used in variant selection affected the detected frequency of antigenic variants at least in some instances, (c) different frequency of antigenic variants were detected by monoclonal antibody XY103 when different assay systems (antibody present only during pre-inoculation period versus antibody present both during pre-inoculation and post-inoculation periods) were employed.

(II) Idiotype of clonal responses to influenza virus hemagglutinin.

Syngeneic and homologous anti-idiotype antisera were used to study the clonal responses of mice to influenza virus hemagglutinin. Extensive cross-reactive idiotypy was demonstrated among monoclonal antibodies to distinct antigenic determinants on PR8 hemagglutinin. Analysis of idiotypes on monoclonal antibodies to B/Lee HA revealed: (a) a true individual idiotype not shared by any other monoclonal antibody, (b) idiotypic determinants shared by a few monoclonal antibodies, and (c) cross-reactive idiotypes which could be detected on all of the monoclonal antibodies tested. In addition, when the idiotypes expressed during primary and secondary responses of BALB/c mice immunized with influenza B/Lee virus were examined it was found that some cross-reactive idiotypes

were expressed during primary and secondary responses whereas others were detected only in the primary or secondary response.

Furthermore, it was found that this cross-reactive idiotype is not present at birth, but appeared shortly (7 days) after birth and persists in adult mice. When sera from various strains of mice obtained before and after immunization with B/Lee virus were tested for antiviral antibody and expression of IdX, low levels of cross-reactive idiotype were detected in the sera of all strains following primary and/or secondary immunization. These results demonstrate that the expression of IdX following immunization with B/Lee virus is not under Igh-C or MHC gene control.

When the expression of this IdX in mice immunized with natural variants of influenza B/Lee virus was studied, similar low levels of cross-reactive idiotype were also demonstrated. This suggests that the same germline genes are employed in the responses to variants and to B/Lee virus.

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V. AIMS

More than a thousand fold difference of frequencies of antigenic variants detected by monoclonal antibodies was reported both from different laboratories and within the same laboratory. One main purpose of my research was to elucidate the possible explanations for these discrepancies in the frequency of variants detected by different monoclonal antibodies. To address this question, a series of monoclonal antibodies specific for PR8, X31 and B/Lee viruses specific for the same (or overlapping) antigenic determinant(s) and/or against different determinants on the hemagglutinins of the respective viruses were used in these studies. In addition, the same monoclonal antibody was tested with wild type and variant viruses to study the effect of antibody avidity on detected frequency of antigenic variants. Furthermore, serial dilutions of ascites fluid containing monoclonal antibody and different assay systems were also employed in the studies.

The second aim of the research was to investigate the clonal basis of immune responses to influenza viral HA by studying the idiotypy of monoclonal antibodies and the diversity of these clonal responses to influenza virus HA. To address this question, anti-idiotype antisera raised in syngeneic (BALB/c mice) and homologous (A/J mice) systems were employed to study: (i) cross-reactive idiotypes among monoclonal antibodies either against overlapping or different antigenic determinants on PR8 and B/Lee virus HA, (ii) the expression of these idiotypes during primary and secondary antiviral responses of BALB/c mice, (iii) the ontogeny of the cross-reactive idiotype response in BALB/c mice, (iv) whether this cross-reactive idiotype response is under Igh-C or MHC

control, and (v) the idiotypes expressed by BALB/c mice in response to immunization with natural variants of B/Lee virus.

VI. INTRODUCTION

A. GENERAL FEATURES AND CLASSIFICATION OF INFLUENZA VIRUSES:

Influenza viruses are classified as myxoviruses, which are small (80-120 nm in diameter) enveloped animal viruses containing single-stranded, segmented RNA genome of negative polarity (reviewed by Choppin and Compans, 1975). The influenza virions are composed of 1-2% RNA, 68-70% protein, 20-25% lipid and 5-8% carbohydrate (reviewed by Nayak, 1977).

The external surface of the virions is covered by spike-like glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA, comprises more than 90% of the spikes (Drzeniek, et al., 1968), is involved in the attachment to the host cell surface (Lazarowitz and Choppin, 1975) and probably also in fusion to lysosomal membranes (White, et al., 1982) during the initial stage of virus infection. NA, comprises about 10% of the spikes, is involved in the release of progeny virus particles from infected cells (Seto and Rott, 1966) and the prevention of aggregation of newly formed viral particles (Palese, et al., 1974). Beneath the spikes are the host-derived lipid bilayer membrane and underlying matrix (M) protein layer. M proteins are the most abundent protein in the virion (33% of the total protein) but are found in only relatively low concentrations in infected cells, suggesting that synthesis of M protein may be a rate-limiting step in virion maturation (Lazarowitz, et al., 1971). The core component of the virion, ribonucleoprotein (RNP) complex, is composed of nucleoprotein (NP), 3 polymerase (P) proteins and 8 genomic RNA segments in the case of influenza A and B viruses, and 7 segments in he case of influenza C virus (reviewed by Choppin and Compans, 1975). Ulmanen, et al. (1981) recently suggested that PB2

protein recognizes the 5'-terminal cap 1 structure ($m^7G_{ppp}N_m$) on RNAs, and PB1 protein initiates transcription via the incorporation of a guanosine residue onto the 3' end of a capped RNA fragment. The molecular weights of influenza A virus RNA segments, their corresponding gene products and the functions of these proteins where these are known are summarized in Table I.

Influenza viruses are classified into 3 types (A,B and C) according to serological relatedness of their internal proteins. Thus, the nucleoprotein and M proteins of all influenza A virus strains cross-react serologically, whereas there is no cross-reaction among influenza A, B and C virus internal proteins (Pereira, 1969). Type A viruses, unlike influenza B and C viruses, can infect and be transmitted in nature among animal species other than man, and are associated with pandemic diseases (reviewed by Kilbourne, 1975). Like influenza A virus, influenza B virus also contains 8 RNA segments whereas only 7 segments have been detected in influenza C virus (Racaniello and Palese, 1979). In addition, it should be noted that neuraminidase is found in influenza A and B viruses, but not in influenza C virus (Kendal, 1975; Racaniello and Palese, 1979).

Influenza A viruses are subdivided into subtypes according to cross-reactivity of their surface glycoproteins in double immunodiffusion test (Shield, et al., 1980). The 12 hemagglutinin subtypes are:

H1 (previously HO, H1, H_{SW1})

H2 (previously H2)

H3 (previously H3, Heq2, Hav7)

H4 (previously Hav4)

H5 (previously Hav5)

H6 (previously Hav6)

H7 (previously Hav1, Heq1)

H8 (previously Hav8)

H9 (previously Hav9)

H10 (prviously Hav2)

H11 (previously Hav3)

H12 (previously Hav10)

The nine neuraminidase subtypes are:

N1 (previously N1)

N2 (previously N2)

N3 (previously Nav2, Nav3)

N4 (previously Nav4)

N5 (previously Nav5)

N6 (previously Nav1)

N7 (previously Neq1)

N8 (previously Neq2)

N9 (previously Nav6)

The subtypes of influenza A viruses that infect humans are: (reviewed by Schulman, 1978; Young and Palese, 1979; Bean, et al., 1980)

New Grouping System	1971 System	Years
H1N1	H0N1	1929-1946
H1N1	H1N1	1946-1957
H2N2	H2N2	1957-1968
H3N2	H3N2	1968-present
H1N1 (reappearance)	H1N1 (reappearance)	1977-present
recombinants of	recombinants of	1978-present
H1N1 and H3N2	H1N1 and H3N2	

TABLE I

MOLECULAR WEIGHTS (IN DALTONS) OF INFLUENZA A VIRUS RNA SEGMENTS,

THEIR CORRESPONDING PROTEINS AND FUNCTIONS OF PROTEINS

Segment	molecular weight of vRNA(10^{-4})[a]	gene product(s)	molecule weight of viral protein(10^{-3})[b]	function of viral protein
1	89	PB2	87	the smaller of the two basic P proteins; recognizing the 5'-terminal cap structure on RNAs.(Ulmanen,1981)
2	89	PB1	89-96	the larger of the two basic P proteins; the initiation of viral RNA transcription (Ulmanen, 1981).
3	86	PA	80-83	acidic P protein; elongation of the viral mRNA (?) (Ulmanen, 1981).
4	66	HA	75-80 HA_1=49-60 HA_2=23-30	major surface glycoprotein; attachment to the host cell surface; Ab to HA is neutralizing. (Lazarowitz and Choppin, (1975).
5	56	NP	55-65	major component of ribonuclear protein complex; may be responsible for stability of RNP complex.
6	48	NA	55-70	minor surface glycoprotein; involved in the release of progeny viral particles from infected cells and prevention of aggregation of newly formed viral particles(Seto and Rott,1966;Palese et al. (1974).
7	28	M1=21-27[c] M2=15		matrix protein underneath the viral envelope; step-limiting component in virion maturation (Lazarowitz et al., 1971).
8	21	NS1 23-30[d] NS2 11-14		nonstructural proteins;function unclear

(TABLE I)

a. Values are from Desselberger and Palese, 1978.

b. Values are from Inglis et al. 1976; reviews of Nayak, 1981 and Choppin and Compans, 1975.

c. M_1 and M_2 are overlapping proteins which are coded by the same M gene via different reading frames (Winter and Fields, 1980; Allen et al. 1989; Lamb et al. 1981).

d. Both NS_1 and NS_2 are coded for by the smallest vRNA segment. The coding regions of NS_1 and NS_2 mRNA are in different reading frames. (Lamb et al. 1978; Lamb and Lai, 1980).

B. SPECIFIC IMMUNE RESPONSES TO INFLUENZA VIRUS INFECTION.

There is abundant evidence that specific antibody to HA present at the time of virus challenge is protective, that immune subjects are less likely to become infected, and if infected develop less serious disease than non-immune subjects. Moreover, passive immunization with specific antibody at the time of challenge has been shown to be protective. Hence there is little doubt that anti-HA antibody exerts a significant effect at least early in infection. In addition, antibody to neuraminidase although lacking neutralizing activity has also been shown to confer protection early in infection (reviewed by Schulman, 1975).

In addition to humoral responses, cell mediated immune (CMI) responses are also elicited by influenza virus infection and to some extent following immunization with formalin inactivated virus. To begin with, antibody response to influenza virus HA is T dependent (Virelizier, et al., 1974). In addition, MHC-restricted cytotoxic T cell responses (Yap and Ada, 1978), increased NK cell activity (Wyde, et al., 1978), antibody dependent cellular cytotoxicity (ADCC) (Greenberg, et al., 1979), blastogenic responses to influenza virus antigens in vitro (Dolin, et al., 1978), and delayed type hypersensitivity reaction (Habershon, et al., 1973) all have been demonstrated, but their contribution to recovery from infection or pathogenesis of disease is still controversial.

Following infection with influenza virus, athymic nude mice make little antibody, develop much less pulmonary inflammation, and survive longer than normal mice. On the other hand, virus is cleared less rapidly and is more often disseminated to other organs in nude mice (Wyde, et al., 1977; Sullivan, et al., 1976). Administration of anti-lymphocyte serum (ALS) to mice infected with influenza virus results in

reduced lung lesions and increased survival in association with decreased lung virus titers and increase in HI antibody titers (Suzuki, et al., 1974). Cyclophosphamide (an anti-inflammatory drug), when injected into mice, (a) converted a relatively benign infection with an avirulent strain of influenza virus into a severe infection associated with extensive pneumonia and death, and (b) increased the mortality rate of mice infected with low concentrations of virulent virus, but lengthened the survival time of mice infected with high concentrations of the same virulent strain (Hurd and Heath, 1975). Schulman et al. (1977) showed (a) that treatment of influenza virus infected mice with anti-thymocyte serum (ATS) resulted in slower virus clearance and diminished serum anti-HA antibody titers, and (b) that adoptive transfer of sensitized spleen cells resulted in a more rapid virus clearance in the recipients. Yap and Ada (1978) also showed that the adoptive transfer of secondary immune cytotoxic T cells led to more rapid reduction of influenza virus titers in mouse lungs and protected mice from death. Moreover, these latter authors demonstrated that the effector cell population which conferred this effect were Ly $2^{+}3^{+}$ and required compatability at the K or D end of the H2 locus, and hence shared characteristics in common with cytotoxic T cells (Yap and Ada, 1978).

Taking together all the above experiments: The T cell dependency of antibody response to influenza virus HA is confirmed. These results also suggest that cytotoxic T cells probably contribute significantly to virus clearance, and that CMI responses might contribute either to recovery or to pathogenesis of disease depending on the methods employed and the dose and the virulence of the virus used in challenge.

C. GENETIC VARIATION OF INFLUENZA VIRUSES.

Although influenza virus was first isolated from fowl plaque in 1901, from pigs (Shope, 1931) in 1931 and from humans (Smith, et al., 1933) in 1933, and has been studied extensively since then, control of pandemic and epidemic influenza has not been achieved. To a great extent, this failure reflects the unusual capacity of influenza viruses in nature to change their surface proteins, HA and NA. Two kinds of changes have been classified, antigenic shift and antigenic drift (Webster and Laver, 1975). Antigenic shifts have been observed thus far only in influenza A viruses and occur approximately every 10 to 20 years. It has been suggested that these variants arise by recombination of human strains and influenza A virus resident in animal or avian species (Kilbourne, 1968; Webster and Laver, 1975; Scholtissek, et al., 1978) or hypothetically by reemergence of an 'old' strain (Nakajima, et al., 1978). Analysis of H2N2 and H3N2 strains by RNA hybridization has provided evidence that the former contained four genes (P1, P2, P3, NP) derived from antecedent H1N1 virus and that the latter derived all of their genes except the HA gene from H2N2 virus. These data are in accord with the recombinational theory of the appearance of new pandemic strains (Scholtissek, et al., 1978). On the other hand, the reappearance in 1977 of a strain of H1N1 virus almost identical in all its genes to strains in circulation in 1950 is compatible with a recycling model, although alternate explanations for the latter event have been advanced (Nakajima, et al., 1978). In any event, once antigenically noval strains have appeared, preexisting antibody to older strains in the human population is no longer effective and pandemic disease is observed.

Antigenic drift during the intrapandemic period has been observed in type A influenza viruses. The process is thought to involve stepwise mutations which result in gradual changes in the antigenic structure of viral surface proteins. Subsequent selection in the presence of antibody to the previous strain favors the growth of antigenic variants (Webster and Laver, 1975; Laver, et al., 1979).

Different techniques, such as nucleic acid hybridization (Scholtissek, et al., 1978; Palese, et al., 1978), oligonucleotide mapping (Nakajima, 1978; Young, et al., 1979; Young and Palese, 1979), nucleotide sequencing (Porter, et al., 1979; Winter, et al., 1981) peptide mapping (Laver and Webster, 1972), peptide sequencing (Air, et al., 1981) and monoclonal antibodies (Yewdell, et al., 1979; Lubeck, et al., 1980) have been used to investigate genetic changes in influenza viruses.

cDNA-RNA hybridization analysis reveals that variation among genomes of type A strains is more extensive than among genomes of type B strains, and genomes of type B strains vary more than those of type C strains (Palese, et al., 1981). Peptide sequence data shows that the rates of genetic drift for the matrix and non-structural proteins are not slower than those of the HA protein within the same HA subtypes. Therefore, antibody selection does not seem to play a role in the rate of amino acid change during intrapendemic period (Air and Hall, 1981).

Generalizations drawn from comparisons concerning amino acid sequence identity and nucleotide sequence identity among different subtypes of influenza A viruses (A/PR/8/34(H1), A/Jap/57(H2), A/Mem/102/76(H3), and A/Fowl Plague Virus/Rostock(H7)) can be seen in the

following table (Brownlee, 1980; Winter, et al., 1981; Gething, et al., 1980; Verhoeyen, et al., 1980):

	% Nucleotide Conservation		% Amino Acid Conservation	
Comparison	HA1	HA2	HA1	HA2
H1/H2	61	72	58	79
H1/H3	45	58	35	53
H1/H7	44	58	33	51
H2/H3	45	57	36	50
H2/H7	46	59	35	53
H3/H7	45	66	36	65

H1 and H2 are more homologous to one another than to other subtypes tested here; this suggests they diverged more recently from one another than other subtypes. HA1 shows more variability among subtypes than HA2 in all cases. Thus the discrimination of subtypes is primarily dependent on HA1.

Furthermore, from comparisons of the NH_2-terminal region of HA1 (20% of the whole HA sequence) of 32 virus strains, Air (1981) was able to construct a proposed evolutionary tree among 12 hemagglutinin subtypes (Figure 1).

Although HA molecules of different subtypes of A viruses (Winter, et al., 1981; Gething, et al., 1980; Verhoeyen, et al., 1980) and B virus (Krystal, et al., 1982) show a great degree of sequence variability and length difference, the sequences required for structure and function of HA appear to remain relatively unchanged. These include a hydrophobic signal peptide, the amino terminus of HA2 (which may be involved in fusion of viral and host membranes (White, et al., 1981)), hydrophobic

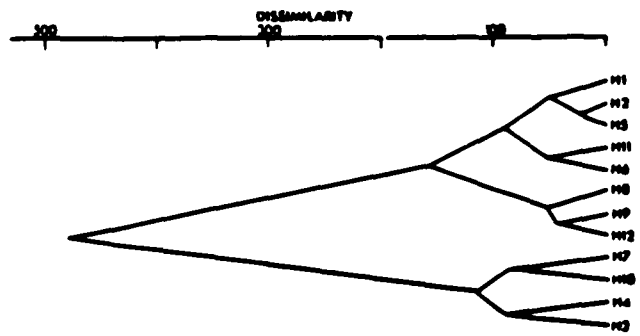

Figure 1. Sequence relationships among the 12 HA subtypes (from G.M. Air, 1981)

amino acids at the carboxy terminus of HA2 (which fix HA in the viral membrane), most cysteine residues (providing disulfide bonds required for proper conformation of HA) and many proline residues (which presumably influence the secondary structure of HA), and an HA1-HA2 cleavage site involving an arginine residue.

Since many genetic changes in the HA and NA genes do not result in detectable antigenic changes, serologic methods must be included in the analysis in order to detect changes associated with antigenic variants. In this respect, monoclonal antibodies have a distinct advantage over heterologous immune sera in that they can be applied to detect specific virus variants restricted to a particular antigenic determinant. So far, monoclonal antibodies to influenza HA molecules have been raised in several laboratories to map the amino acid changes at the antigenic sites (Laver, et al., 1979; Webster and Laver, 1980) and to study the frequencies of antigenic variants (Laver, et al., 1979; Yewdell, et al., 1979; Lubeck, et al., 1980; Portner, et al., 1980).

The initial structural studies on variants selected with monoclonal antibodies were primarily focused on the H3 subtype for which amino acid sequence data was available. Laver, et al. (1979) used monoclonal antibodies against A/Memphis/1/71(H3N2) to select antigenic variants which were then analyzed by peptide mapping to determine specific amino acid substitution associated with antigenic sites. They demonstrated that there were at least 3 antigenic areas on H3 HA molecules, and that most variants they selected showed a single amino acid substitution in HA1 and no sequence changes in HA2. Additional nucleotide sequence data on natural variants of H3 hemagglutinin revealed a pattern of clustering

of substitutions in four regions of the HA molecule (Both and Sleigh, 1981).

With the availability of primary sequence data and data obtained by x-ray crystallography, Wiley, et al. (1981) were able to construct a three dimensional model of the 1968 H3 hemagglutinin. Furthermore, after localizing the substitutions in natural and laboratory selected variants they proposed that there were four antigenic regions (A, B, C and D) on the H3 hemagglutinin. The three dimensional structure of the HA of other subtypes is not available. However, other investigators (Yewdell, et al., 1979; Gerhard, et al., 1980) could identify at least four non-overlapping antigenic regions on PR8 (H1N1) HA using a large panel of monoclonal antibodies.

Yewdell, et al. (1979) using monoclonal antibodies to influenza A/PR/8/34 (H3N2) virus detected antigenic variants in an 'allantois on shell' system and reported that the frequencies of antigenic variants for 3 different determinants of the H1 hemagglutinin were in the range of $10^{-5.5}$ to $10^{-6.2}$. Frequencies of $10^{-4.1}$ to $10^{-5.0}$ were reported for A/Memphis/1/71 (H3N2) virus detected either in embryonated hen's eggs or in the Madin-Darby canine kidney (MDCK) cell system (Laver, et al., 1979; Portner, 1980). When Lubeck et al., (1980) used monoclonal antibodies against the hemagglutinins of A/PR/8/34 (H1N1) virus X31 virus (an H3N2 recombinant virus of A/Aichi/2/68 and A/PR/8/34 (H3N2) virus) and B/Lee/40 virus to analyze the frequencies of antigenic variants in MDCK monolayers, a wide range ($10^{-4.4}$ to $<10^{-8.8}$) was found. Thus, discrepancies in frequencies of variation were observed both among different laboratories and within the same laboratory, and several explanations which could contribute to these differences were proposed

(Lubeck, et al., 1980): (a) differences in viability or growth potential of different variants in the cell culture system used; (b) differences in mutational frequencies at different antigenic sites; and (c) differences in avidity among monoclonal antibodies used in selection. One of the main purposes of this thesis is to elucidate the possible reasons that may cause these differences in detected frequencies of antigenic variants in a cloned virus population.

D. GENERAL FEATURES OF IDIOTYPES.

Idiotypes were first described by Kunkel et al. (1963) in human immunoglobulins, and by Oudin and Michel (1963) in rabbit immunoglobulins. Originally defined by Oudin (1966) as 'the antigenic specificities of antibodies.' Different idiotypes could be identified both among antibodies of one individual against different antigens and among antibodies of different individuals against the same antigen. In short, idiotype is the individuality of antibody specificity.

The reagents used to detect idiotypes are generally anti-idiotype antisera, which can be raised in the following four ways:

(1) Autologous anti-idiotype antisera: these are obtained by injecting antibody back into the same individual (autoimmunization). This method was first described by Rodkey (1974): The rabbit was immunized by polysaccharides, and the antibodies collected from this rabbit were injected into the same rabbit two years later when anti-polysaccharide antibody in sera could no longer be detected.

(2) Syngeneic anti-idiotypic antisera: these are obtained by injecting antibodies into the same inbred strain of animals (isoimmunization). Generally syngeneic anti-id antibodies are relatively difficult to obtain especially when the antibodies share idiotypic determinants with

'natural' antibodies (Bona, 1981). Modified immunization procedures are used to generate this kind of antisera. Using T15 myeloma protein (BALB/c origin), Cosenza, et al. (1979) prepared anti-T15 antibodies by injection into T15-idiotype suppressed BALB/c mice. Bona et al. (1979a) prepared anti-id antibody by hyperimmunizing syngeneic mice with multiple injections of keyhole limpet hemocyanin (KLH) conjugated antibody.

(3) Homologous anti-idiotypic antisera: these are obtained by injecting antibodies into different strains of animals of the same species (alloimmunization). This kind of anti-id sera are relatively easy to obtain, and can be prepared simply by immunization with antibodies emulsified in Freund's complete adjuvant. The prepared antisera have to be extensively adsorbed with normal Ig or other myeloma proteins of the strain from which the idiotypes were obtained to remove anti-allotype components.

(4) Heterologous anti-idiotypic antisera: these are obtained by injecting antibodies into different species (heteroimmunization), and are easy to obtain. To make the antisera idiotype specific, they have to be adsorbed with normal Ig or with other myeloma proteins to remove anti-isotype and anti-allotype antibodies.

Following the discovery of idiotypes, localization of idiotypic determinants has been extensively investigated during the past two decades. Idiotypic determinants were first localized on Fab fragment in human Ig by Kunkel et al. (1963) and in rabbit Ig by Grey et al. (1965). Wells et al. (1973) showed that the isolated Fv fragment of mouse myeloma protein MOPC 315 could inhibit the reaction between complete myeloma protein and anti-idiotypic sera. This demonstrated that idiotypic determinants are in the V region domain of antibodies. The relationship

between idiotypic determinants and the antigen combining site was first studied by Breut and Nisonoff (1970). They showed that the hapten (benzoate) inhibited the binding of anti-idiotypic sera and anti-benzoate antibody. This suggested that either idiotypic determinants are associated with the antigen binding site of the antibody, or that the hapten can cause a conformational change or steric hindrance around the idiotypic determinants. The close relationship between idiotypic determinants and the antigen binding site was also demonstrated in many other systems, including myeloma proteins specific for DNP and TNP (Sirisinha and Eisen, 1971), phosphocholine (Sher and Cohn, 1972), α (1->3) dextran (Carson and Weight, 1973), β (1->6) D-galactan (Potter, et al., 1977) and (2->1) fructosan (Lieberman, et al., 1975). However, there are a few exceptions: Claflin and Davie (1975a) demonstrated that phosphocholine did not inhibit the binding of A/J anti-T15IdX sera and T15Id. Helman et al. (1976) found that rabbit anti-MOPC 315Id sera primarily interacted with framework associated idiotypes and BALB/c syngeneic anti-MOPC 315Id sera mainly interacted with the antigen binding site.

Comparison of amino acid sequences of cross-reactive idiotype positive and cross-reactive idiotype negative heavy (H) (Capra and Kehoe, 1974; Capra, et al., 1975a; Capra, et al., 1978) or light (L) (Capra, et al., 1975b; Capra, et al., 1977; Capra, et al., 1978) chain variable regions illustrated that the differences between Id^+ and Id^- heavy or light chains resided primarily in their hypervariable regions.

Lieberman, et al. (1977) separated and reconstituted H and L chains of inulin-specific myeloma proteins, and found that some idiotypic specificities found on intact immunoglobulin molecules can be localized

to either the light or heavy chain, but that idiotypic specificities in general require the interaction of both chains.

Clevinger, et al. (1980) compared amino acid sequences and idiotype expression of 12 anti-α-(1->3) dextran binding proteins. Interestingly they found cross-reactive idiotypes were associated with V region determinants within the second hypervariable region, whereas the individual idiotypes were associated with the D-segment in the third hypervariable region.

In summary, idiotypes are phenotypic markers in the V region of H or L chains, or both. They generally are associated with antigen binding sites of antibodies (with few exceptions), and amino acid sequence analysis studies have localized them primarily to hypervariable regions.

E. CROSS-REACTIVE IDIOTYPES.

Although according to Oudin's original definition of idiotypes, each antibody could express only one individual idiotypic specificity, studies of idiotypes expressed on myeloma proteins illustrated that antibodies to the same antigen from different individuals of an inbred strain could share cross-reactive idiotypes. Thus, each antibody can express two major categories of idiotypes (Bona, 1981):

(1) Individual idiotype (i.e. Private idiotype, IdI): This is the idiotype of Oudin's classical definition. It is characteristic of one antibody or one myeloma protein, and generally it is not shared by other antibodies or by other myeloma proteins.

(2) Cross-reactive idiotype (i.e. Common or public idiotype, IdX): This idiotype is usually shared by antibodies and myeloma proteins exhibiting the same antigenic specificities obtained from different individuals of

inbred strains. It is rarely shared by antibodies with the same specificity produced by individuals among outbred strains.

According to the distribution of idiotypes among different strains or different species of mammals, cross-reactive idiotypes can be further classified into three groups (Bona, 1981):

(1) Allotype linked interstrain IdX's: These IdX are expressed only in strains of mice that bear a particular allotype (i.e. Igh-C genes) regardless of other genetic markers. They segregate with allotypes in a Mendelian fashion. The typical examples are:

(a) IdX's A, B and G of inulin-binding antibodies: IdX's A, B and G are shared by a group of myeloma proteins specific for polyfructans (i.e. inulin and bacterial levan (BL)) and anti-inulin antibodies produced in some mice immunized with BL and inulin (Lieberman, et al., 1975). The expression of these IdX's are linked to the Ig-1^a allotype locus (Lieberman, et al., 1976).

(b) IdX of anti-Ars: The expression of certain IdX of anti-Ars (p-Azophenylarsonate) antibodies is associated with the Ig-1^a allotype (Nisonoff, et al., 1977).

(c) S117 IdX: The S117 IdX is a cross-reactive idiotype among antibodies to group A streptoccal carbohydrate (A-CHO). The expression of S117 IdX is under the control of a gene which is linked to the Ig-1^a allotype locus in BALB/c mice and in other strains carrying the same Ig-1 haplotype (Berek, et al., 1976).

(d) ASA IdX: The ASA IdX is a cross-reactive idiotype shared by myeloma proteins specific for N-acetylglucosamine, the dominant sugar of A-CHO antigen and anti-streptococcus A antibodies elicited in A/J mice.

The expression of this A5A IdX is associated with the Ig-1^e allotype of mice (Eichmann, 1973).

(e) J558 IdX: The J558 IdX is shared by J558 and MOPC 104E myeloma proteins, and anti-α (1->3) dextran antibodies in BALB/c mice. The expression of this IdX is linked to the Ig-1^a allotype in mice (Carson and Weigert, 1973).

(2) Interstrain IdX not linked to allotypes: Some idiotypes are expressed in various strains of mice independently of allotype or other genetic markers. Examples of these idiotypes are:

(a) IdX of β-6-GALBMP: Antibodies induced in mice immunized with gum ghatti share cross-reactive idiotypes with β-6-GALBMP, a β-1,6-D-galactan binding myeloma protein. This β-6-GALBMP IdX is detected in various strains of mice either bearing Ig-1^a or Ig-1^b allotypes. The expression of the IdX of β-6-GALBMP in different strains and during different stages after immunization is very heterogeneous. Thus, it is speculated that some of this diversity may arise from somatic mutations rather than from germ line genes (Mushinski and Potter, 1977).

(b) IdX of anti-IgG antibodies: Kunkel et al. (1973) using hemagglutination and hemagglutination inhibition assays found that IgM proteins with anti-γ-globulin activity from different individuals with the so-called 'mixed cryoglobulin' syndrome share cross-idiotypic specificity.

(c) IdX of anti-$IgG_{2a}{}^b$: Bona et al. (1980) showed that a cross-reactive idiotype expressed on anti-allotype antibodies to the b allelic forms of IgG_{2a} ($IgG_{2a}{}^b$) is regulated by major histocompatibility complex (MHC)-encoded immune response (Ir) genes.

(d) GA-1 IdX: The GA-1 idiotype is expressed in a minor fraction of the anti-GAT (synthetic polymer poly-(Glu^{60}, $L\text{-}Ala^{30}$, $L\text{-}Tyr^{10}$) antibodies, and is specific for 'GA' related antigenic determinants. The identification of this idiotype was based on a heterologous idiotypic interaction between guinea pig anti-idiotype antiserum made against pooled purified D1.Lp anti-GAT antibodies and a monoclonal hybridoma anti-GAT antibody specific for the GA antigenic moiety. This idiotype was detected in the 21 inbred strains of mice studied. Hence it is an interstrain cross-reactive idiotype (Ju, et al., 1979).

(3) Interspecies IdX's: Some IdX are found in antibodies produced by different animal species. Examples are:

(a) CGAT IdX: The CGAT idiotype is present on the major fraction of anti-GAT antibodies, and is induced by the 'GT' related antigenic determinant on GAT molecules. This idiotype is also expressed in antibodies to GT, GAT and (T,G)-A-L synthetic polymers (i.e. polymers containing glutamic acid and tyrosine) (Ju and Dorf, 1979). The expression of the CGAT idiotype in mice is independent of Ig-1, H2 and V_h genetic markers (Ju, et al., 1978a). In addition, the CGAT Id has also been identified in 9 of the 13 inbred strains of rats tested (Ju, et al., 1978b).

(b) Idx of anti-PC antibodies: HOPC 8 (i.e., H8), one of the PC (phosphocholine)-binding myeloma proteins which shares the T15 Idx (Lieberman, et al., 1974), is present on anti-PC antibodies of all mice regardless of histocompatibility or allotype markers. Furthermore, H8Id was found in one out of eight hamsters immunized with PC (Claflin and Davie, 1974).

Riesen (1979) investigated the idiotypic cross-reactivity of mouse and human monoclonal immunoglobulins with PC-binding activity. Among the 3 anti-PC myeloma proteins of mice studied, he found that there is a weak idiotypic cross-reactivity between the PC-binding human IgM_{PR} and the mouse IgA MOPC 167 and MOPC 603, while the murine IgA TEPC 15 does not share any idiotypic determinant in common with the human protein IgM_{PR}.

(c) Val IdX: The antibody population which binds human sickle cell hemoglobin (HbS) but not normal human hemoglobin (HbA1) is called anti-VA1 antibody. Karol et al., (1978) compared the idiotypic cross-reactivity among anti-VA1 antibodies raised in goats, sheep and guinea pigs using anti-idiotypic antisera produced in rabbits immunized with the goat and sheep antibodies. Idiotypic cross-reactivity was found between goat and sheep anti-VA1 antibodies, but guinea pig anti-VA1 did not share idiotypic cross-reactivity with sheep or goat anti-VA1 antibodies.

Although idiotypes are commonly shared by antibodies with the same antigen binding activity, several unexpected instances of sharing of idiotypes have been observed: Oudin and Conzenave (1971) observed shared idiotypes among different antibodies against hen ovalbumin, among non-cross reactive antibodies to ovalbumin from different animal species, or even among antibodies of unknown specificities. Karol, et al. (1978) demonstrated cross-reactive idiotypes among sheep antibodies against different determinants of the same human hemoglobin molecule. Bona et al. (1979) found that 1-2 week old BALB/c mice express a cross-reactive anti-inulin idiotype on antibody molecules that do not bind to inulin. Ju et al. (1980) demonstrated shared idiotypes among antibodies which bear distinct binding specificities with a diverse series of synthetic polypeptides. Hence, cross-reactive idiotypes do exist in certain cases

among antibodies against different antigenic determinants on the same molecule, among antibodies against different antigenic determinants on the same molecule, among antibodies against different antigen molecules, and among antibodies of unknown specificities. The mechanisms which possibly lead to cross-reactive idiotypes will be dealt with in the Discussion section.

F. ONTOGENY OF IDIOTYPE EXPRESSION.

The ontogeny of humoral immune responses to various antigens has been extensively studied in different species, such as the chickens (Wolfe, et al., 1957), mice (Goidl, et al., 1974; Press and Klinman, 1974; Klinman and Press, 1975), rats (Halliday, 1957; 1964), guinea pigs (Baer, 1963), rabbits (Bellanti, et al., 1963), pigs (Segre and Kaeberle, 1962a; 1962b), lambs (Sterzl and Silverstein, 1967; Silverstein, 1977) and children (Rannon, et al., 1960; Lennette, et al., 1962). The general conclusions from these studies are: (a) There is more restricted heterogeneity with respect to antibody responses in fetuses or neonates than in adults. (b) Not all precursors of lymphocytes producing antibody to different antigens mature at the same time during ontogenic development. The acquisition of immune responses to various antigens during ontogeny is independent of environmental influences and is highly ordered. For example: Klinman and Press (1974; 1975) using an <u>in vitro</u> limiting cell dilution splenic focus technique demonstrated in mice that precursors of B cells for 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) were already present in high frequencies at birth, and attained adult frequencies within one week after birth, while precursors for fluorescein (FL) and phosphorylcholine (PC) were not detected until 5 days after birth. Silverstein (1977) found that in the

lamb, antibody responses against certain viruses, ferritin, ovalbumin and hemocyanin could be detected during the fetal stage, whereas antibody responses to diphtheria toxoid, the O antigen of Salmonella typhosa, and BCG were not detected until 40 days after birth.

Since idiotypes are phenotypic markers of V region genes of immunoglobulin (Weil, et al., 1973), they reflect the expression of relatively restricted clonotypes of B cells and provide insights into the mechanism of B cell diversification. Therefore, the ontogeny of idiotypes has been actively investigated in several laboratories. The following is intended to provide some examples of such studies:

(a) E109 IdX response of anti-BL antibodies: BALB/c mice immunized with bacterial levan (BL), a T-independent antigen that consists of β 2->6 linked polyfructose with β 2->1 branch points (Feldman, et al., 1975), produce at least two groups of antibodies. One group binds inulin (a β (2->1) linked polyfructose) and bacterial levan (BL), and the majority of members of this group express the cross-reactive idiotype E109 IdX (Lieberman, et al., 1975). The other group of anti-BL antibodies is specific for the B (2->6) linkage of BL, and does not express the E109Id.

Studies of BALB/c mice immunized with BL by Bona et al. (1978; 1979) revealed that all BALB/c mice younger than 4 weeks of age mounted a response specific for β (2->6) polyfructose but lacked a response specific to inulin and did not produce antibodies which expressed the E109IdX. Furthermore, this relatively late expression of the response to E109 IdX was attributable to a delay in maturation of precursor B cells specific for the E109 IdX, rather than to the absence of precursor cells, or to a defect in maturation of accessory cells.

(b) X24 IdI and X24 IdX of anti-galactan responses: X24 IdI and X24 IdX (XRPC24 is a galactan-binding myeloma protein) are expressed in adult BALB/c mice immunized with gum ghatti (Mushinski and Potter, 1977). This idiotype has also been detected in the sera of 1-day-old BALB/c mice immunized with gum ghatti (Bona, 1981b).

(c) 384Id$^+$ anti-LPS response: MOPC 384 is a myeloma protein which binds specifically to the d-methyl-D-galactoside determinant of Salmonella tranorea, Salmonella tel-aviv, and Proteus mirabilis. The 384Id was detectable in 1-day-old BALB/c mice immunized with S. tranorea LPS (Bona, 1981b).

(d) T15$^+$ response of anti-PC (phosphocholine) antibodies: Sigal et al. (1976) using an in vitro spleen focus technique demonstrated that among PC-specific clonal precursor cells of adult BALB/c mice, the majority were of the TEPC15$^+$ clonotype. However, the T15$^+$ idiotype does not appear in BALB/c mice until 4-5 days after birth.

(e) IdX response of anti-Ars antibodies: Nutt et al. (1979) demonstrated that B cells expressing the cross-reactive idiotype associated with anti-p-azophenyl-arsonate antibodies were present before day 9 in neonatal mice. The time of appearance of the cross-reactive idiotype was not influenced by genetic background since neonatal A/J and C.AL20 (allotype-congenic of A/J) produced comparable amounts of the cross-reactive idiotype bearing antibodies at the same age.

Cancro et al. (1979) compared the pattern of antigen specificity among monoclonal anti-HA antibody populations from 12-14 day-old and adult mice immunized with influenza A virus, and obtained preliminary results indicating that there were relatively very few responding clonotypes in the young animals as compared to adult animals. Aside from

these observations, at present there are no published data on the ontogeny of specific idiotypes in mice immunized with influenza virus.

G. VARIATION AND PERSISTENCE OF IDIOTYPES DURING THE IMMUNE RESPONSE.

The humoral immune response to most antigens is characterized by a highly heterogeneous population of antibody molecules with different antigen specificities derived from a large array of precursor cell clonotypes. However, the response is much more homogeneous with respect to some idiotypic determinants which could be a direct reflection of response of restricted clonotypes. Furthermore, lymphocyte functions are partially regulated by the network of idiotypes and anti-idiotypes (Bona et al., 1981). Thus, the study of variation and persistence of idiotypes during immune responses will help to elucidate the kinetics of the immune response.

Oudin and Michel (1969) pioneered the study of variation of idiotypes during the immune response. They found that the idiotypic patterns detected in anti-Salmonella typhi sera from different rabbits are different. The idiotype they studied persisted for 29 months in one rabbit, and for 2-5 weeks in two other rabbits. However, in another rabbit, the idiotype was present only at an early stage of immunization.

MacDonald and Nisonoff (1970) studied the variation of a specific idiotype in purified anti-p-azobenzoate antibodies from rabbits, and reported that the idiotype they followed disappeared and was replaced by a new and unrelated idiotype 4 months after immunization. This new idiotype persisted for more than 12 months.

Metzger et al. (1980) observed that the idiotypes of 'early' antibodies and 'late' antibodies expressed in mice immunized with hen's egg lysozyme (HEL) were different.

Bona (1981c) studied the expression of XRPC24 IdI and XRPC24 IdX in BALB/c mice immunized with gum ghatti. He found that both idiotypes lasted for at least 150 days and that the great majority of anti-galactan antibodies expressed XRPC24 IdI and IdX by 90-150 days.

Dzierzak et al. (1980; 1981a; 1981b) investigated the expression of Id460 (MOPC460 is a DNP-binding myeloma protein) in BALB/c mice before and after immunization with 2,4-dinitrophenyl-ovabumin (DNP-OVA) and found that: large amounts (10-100 μg/ml) of $Id460^+$ antibodies that were not specific for DNP were present in normal non-immune mice. During the primary immune response, low levels of Id 460 were detected. However, during the secondary immune response, first a rapid rise and then a rapid fall in the levels of Id460 was observed. This transient pattern of Id460 expression did not parallel the anti-DNP response.

VII. MATERIALS AND METHODS

Mice

BALB/c and CD-1 pathogen-free mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). DBA/2J, C57BL/6, AKR/J, CE/J, C3H/J, CBA/J, DBA/1J, SWR/J, A/J, RIIIS/J, and PL/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). C.B20, BAB.14 and BALB.B mice were kind gifts from M. Porter (NCI). All mice were kept in the animal facility at Mt. Sinai Medical Center, and used between 6 and 15 weeks of age.

Viruses

Influenza viruses A/PR/8/34 (HINI) (PR8 virus), B/Lee/40 (B/Lee virus), a recombinant virus A/Aichi/2/68(H3N2)-A/PR/8/34 (X31 virus) (Kilbourne, et al., 1971), B/Great Lake/54, B/Hong Kong/8/72, B/Victoria/70 and B/Maryland/59 were grown in 10 to 11 day embryonated eggs, and purified on 30-60% sucrose gradients according to standard procedures (Palese and Schulman, 1974). All viruses were stored at -70°C.

Monoclonal Antibodies

Hybridoma cell lines producing monoclonal antibodies against hemagglutinin (HA) of influenza viruses PR8, BLee and X31 were prepared by fusion of spleen cells from BALB/c mice immunized with purified virus and myeloma cells (either P3X63Ag8 or SP2/0) (Kohler and Milstein, 1976; Koprowski, et al., 1977). Briefly, BALB/c mice primed 1 to 3 months earlier by intraperitoneal injection of purified intact virus (10 μg viral protein/mouse) and were reinoculated intravenously with the same amount of virus 3 days prior to fusion. The spleen cells were fused with

myeloma cells at a 10:1 ratio (spleen cell:myeloma cells) in 30% PEG (polyethylene glycol, M.W. = 950-1050, J.T. Baker Chemical Co., Phillipsburg, NJ). The selective HAT (1×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin and 1.6×10^{-3} M thymidine, all from Sigma Chemical Co.) medium was added one day later and the visible hybridoma clones appeared 1-2 weeks after fusion. Supernatant fluids of wells containing hybridomas were screened for antiviral antibody production by radioimmunoassay (RIA) (Rosenthal, et al., 1973) and enzyme-linked immunoabsorbent assay (ELISA) (Voller, et al., 1977). The antiviral antibody positive hybridoma cells were then cloned by the limiting dilution method (McKearn, 1980) and grown as ascites in pristane primed BALB/c mice.

RIA and ELISA Used in Hybridoma Screening

(i) RIA (modified from Rothenthal, et al., 1973): Ninety-six-well polyvinyl chloride plates (Cookes Engineering, Alexander, VA) were coated with purified virus (50 λ of purified virus, 50 μg/ml) at 37°C for 90 min., and saturated with 50% fetal calf serum in phosphate buffered saline (PBS) at room temperature for 90 minutes. After three PBS washings, 50 λ of culture fluid was added and incubated at room temperature for 3 hours. The presence of bound monoclonal antibodies was then determined by applying 50 λ of 50,000 cpm/50 λ of ^{125}I-labeled goat anti-mouse Ig (New England Nuclear, Boston, MA) to each well. After incubation for another 3 hours at room temperature, and extensive washing (15 times) with PBS, the wells were cut and counted in a Beckman γ-counter (Beckman Co., Palo Alto, CA).

(ii) ELISA (modified from Voller, et al., 1977): Polystyrene flat-bottom wells (Dynatech Diagnostics, So. Windham, ME) coated with 100 λ of

purified virus (10 μg/ml HA protein) and then were washed with PBS-tween (Fisher, Pittsburgh, PA) to avoid any subsequent non-specific binding. One hundred lambda of test culture fluid was added to the well and incubated at room temperature for 3 hours. After washing the wells 3 times with PBS-tween, 100 λ of alkaline phosphatase-conjugated affinity purified rabbit anti-mouse I_g (~5 μg/ml) was added. (The rabbit anti-mouse Ig was purchased from New England Nuclear, and conjugated according to a method described by Voller, et al., 1977.) After incubation at room temperature for 3 hours, followed by fifteen washings with PBS-tween, sigma 104 phosphatase substrate (Sigma Chemical Co., St. Louis, MO) dissolved in diethanolamine buffer (Sigma Chemical Co., St. Louis, MO) was added. The enzymatic reaction was allowed to proceed at 37°C in the dark for one hour and stopped by the NaOH, and the color change was read in a multiscan spectrophotometer (Flow Laboratories Inc., Rockville, MD) at OD_{405}.

Assays for Specificity of Monoclonal Antibodies

(a) HA specific monoclonal antibodies

(i) Hemagglutination inhibition assay

Ascites fluids were treated with receptor-destroying enzyme (RDE) (World Health Organization, Atlanta, GA) (Dowdle, et al., 1979), and assayed in a standard HI test (Hierholzer and Sugg, 1969).

(ii) Immunoprecipitation

Some HA specific monoclonal antibodies were also tested in immunoprecipitation assay. Briefly, disrupted ^{35}S methionine labeled virus was mixed with monoclonal antibodies and the mixtures allowed to

bind to Staphylococcus protein A. Bound radiolabeled viral proteins then were identified on SDS polyacrylamide gels.

(b) NA specific monoclonal antibodies:

Two monoclonal antibodies were identified as being NA specific by immunoprecipitation. Various dilutions of these ascites fluids were assayed in a standard NI test (Aymard-Henry, et al., 1973).

(c) Host specific monoclonal antibodies:

Host carbohydrate specific monoclonal antibodies were identified by RIA. Polyvinyl microwells were coated with 50 λ of 50 μg/ml of viral proteins of the virus used during immunization or with another virus of a different subtype. After the wells were saturated with 50% FCS, culture fluids were added and incubated at room temperature for 3 hours. The presence of bound monoclonal antiboides was then determined by applying ^{125}I-labeled goat anti-mouse Ig. Monoclonal antibodies which bound to both viruses were host specific monoclonal antibodies.

(d) NP specific clones, NA specific clones as well as HA specific clones lacking HI activity were identified by gel precipitation technique by P. Graves, J. Young and P. Palese (Department of Microbiology, Mt. Sinai Medical School, NY) were also obtained during the course of research and were not utilized further.

All hybridoma cell lines were kept in 5% DMSO-95% FCS, and stored in liquid nitrogen tank (Cryo-med. Co., Mt. Clmens, MI).

Subclasses

Subclasses of monoclonal antibodies were determined by solid phase radioimmunoassay. Polyvinyl microtiter wells were coated with 50 λ of affinity column purified monoclonal antibodies (100 μg/ml) and then saturated with 50% FCS. The wells were washed 3 times with PBS. Fifty

lambda of 50,000 cpm/50 λ ^{125}I-labeled isotype specific goat anti-mouse antisera (isotype specific goat anti-mouse antisera were generous gifts from P. Mongini, NIH) were added. After incubation at room temperature overnight, the plates were washed with PBS (15 times) and the amount of radioactivity bound to the wells was determined in a Beckman γ-counter. The standards icluded in the assay were MOPC 104 (IgM), anti-Ars (IgG_1), RPC5 (IgG_{2a}), MOPC 185 (IgG_{2b}) and LPS7 (IgG_3).

These results were subsequently verified in an antigen-specific assay of isotype. The wells were first coated with purified virus (50 μg protein/ml) and saturated with 50% FCS. Then either purified or unpurified monoclonal antibodies were added to the wells and incubated at room temperature overnight. After 3 washings with PBS, 50 λ of 50,000 cpm/50 λ of ^{3}H-labeled isotype specific goat anti-mouse antiserum were added, the plates were incubated at room temperature overnight, and washed 15 times with PBS. The amount of radioactivity bound to the wells was measured in a scintillation counter (Beckman Instruments, Inc., Palo Alto, CA).

Purifications of Monoclonal Antibodies by Affinity Column

Virus-coupled affinity columns were made in the following way: Viruses purified on a 30-60% sucrose gradient (Palese and Schulman, 1974) were pelleted in 0.9% NaCl at 25,000 rpm for 90 minutes in a Beckman L2-65B or L3-40 Ultracentrifuge (Palo Alto, CA). The pellets were resuspended in 0.9% NaCl containing 10% octoglucoside (Sigma, St. Louis, MO) for 30 minutes at 37°C, and coupled to cyanogen bromide (CNBr)-activated sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) in bicarbonate buffer (0.1 M, pH 8.3) at 4°C overnight. The remaining

active groups in sepharose were blocked by 1 M ethanolamine in borate buffer (pH 8.0). The absorbent was then washed alternately with low pH (acetate buffer, 0.1 M, pH 4.0) and high pH (borate buffer, 0.25 M, pH 8.0) buffer for 3 cycles to remove uncoupled viral proteins and blocking agent. This virus-sepharose conjugate was stored at 4°C and could be used repeatedly.

The monoclonal antibodies to viral proteins were purified by adsorption of ascites fluid to the virus-sepharose column followed by elution with glycine-HCl buffer (0.1 M, pH 2.8). The purified antibodies were neutralized immediately by addition of saturated Tris buffer and dialyzed against 0.9% NaCl or PBS at 4°C overnight. The columns were regenerated with 10 column volumes of borate buffer (pH 8.0) followed by 10 column volumes of PBS containing 0.02% NaN_3.

Pre-inoculation Neutralizatin and Post-inoculation Plaque Reduction Titers

Both pre-inoculation neutralization and post-inoculation plaque reduction titers were determined in plaque assays performed in the following way: MDCK monolayers were infected with either virus or a mixture of virus and antibody. After one hour period of adsorption, the inoculum was removed and 5 ml of agar overlay containing 1 x MEM (minimum essential medium, Gibco, Grand Island, NY), 0.5% agar and 2 μg/ml trypsin was added. The number of plaques was estimated after 3-4 days of incubation of 37°C in an atmosphere of 5% CO_2.

(i) In the preinoculation neutralization test (Jahiel and Kilbourne, 1966), equal volumes of virus expected to yield approximately 100 PFU (plaque forming units)/0.1 ml and of serial 4-fold dilutions of RDE treated ascites fluid were mixed, and allowed to react at 37°C for 45

minutes. At the end of the reaction period, 0.2 ml of the mixtures were inoculated onto MDCK monolayers. After a one hour period of adsorption, the inoculum was removed, the dishes were washed wth PBS, and a 5 ml of agar overlay was added. The number of plaques was counted after 3-4 days of incubation.

(ii) In the postinoculation plaque reduction test (Jahiel and Kilbourne, 1966) the MDCK monolayers were infected with approximately 100 PFU of virus; after virus adsorption, an agar overlay containing different dilutions of RDE treated ascites fluid was added, and plaques were counted after 3-4 days incubation.

Preinoculation neutralization and postinoculation plaque reduction titers were determined as the reciprocal of the dilutions of ascites fluid that reduced the number of PFU by 50%.

Frequencies of Antigenic Variants Detected by Monoclonal Antibodies

(i) Routine assays: The frequencies of antigenic variants were obtained from the ratios of the number of variants detected in the presence of an excess amount of monoclonal antibodies to the total number of infectious viral particles in cloned virus seeds (Lubeck, et al., 1980). Briefly, wild type viruses were first cloned in MDCK monolayers, and then allantoic fluid seeds were prepared in 10 to 11 day old chick embryos. Antigenic variants were assayed by incubating these virus seeds with RDE-treated ascites fluids containing monoclonal antibodies (45 minutes at 37°C) and plaquing on MDCK monolayers. Visible plaques were picked separately and after preparation of seed viruses in chick embryos, were confirmed to be variants by HI and /or neutralization assay with homologous monoclonal antibodies. The total number of infectious viral

particles in the virus seeds was determined following serial dilutions of virus seeds.

(ii) Frequencies of antigenic variants detected at different monoclonal antibody concentrations: The assay system was the same as (i) except that different dilutions of RDE-treated ascites were tested. In this assay, the plaques picked were screened very carefully by both HI tests and neutralization assay to ensure that none of the virus populations which survived at a particular antibody concentration were unneutralized wild type virus particles.

(iii) Frequencies of antigenic variants detected when antibody was present only during the preinoculation period: This procedure is similar to (i) except that after the adsorption period, the inoculum was removed and the dishes were washed with PBS before the agar overlay was added.

Binding Constants (K)

The binding constants (K) of monoclonal antibodies with viruses were determined by solid-phase RIA (Frankel and Gerhard, 1979; Frankel, 1980). In brief, 96 well polyvinyl chloride microtiter wells were first coated with a constant amount of purified virus (50 λ of 50 μg/ml protein in PBS) at 37°C for 90 minutes, and then uncovered sites on the wells were saturated with 50% FCS. After three washings with PBS, 50 λ of two-fold dilutions containing concentrations ranging from 0.01 μg/ml to 5 μg/ml of affinity purified monoclonal antibodies were added. The plates then were incubated at room temperature overnight to reach equilibrium (Zollinger, et al., 1976). The binding of antibody to the virus coated plates was measured following addition of 50 λ of 50,000 cpm/50 λ of ^{125}I-goat anti-mouse Ig (New England Nuclear, Boston, MA). The concentration of bound antibody was determined by a binding curve established simultaneously

using increasing antigen concentration and constant concentration of antibody, and was determined as the maximum amount of antibody bound at optimal antigen concentration. The concentration of free antibody was calculated by substracting the concentration of antibody bound from that of total antibody. The binding constants (K), which reflect the avidity of binding between antigens and antibodies, were derived from linear regression analysis of a Schatchard plot of [Ab] bound/[Ab] free versus [Ab] bound (Schatchard, 1949; Frankel and Gerhard, 1979).

Quantitation of Monoclonal Antibodies in Ascites Fluids

The concentrations of monoclonal antibodies in ascites fluids were detected by solid-phase RIA modified from a method described by Stevens (1980) and Yarchoan, et al. (1981). Briefly, polyvinyl microtiter wells were filled with 50 µl of 50 µg protein/ml of purified virus for 90 minutes at 37°C. The wells then were saturated by the addition of 50% FCS, and washed 3 times with PBS. Quadraplicate, serial 2-fold dilutions of the ascites fluid being tested then were added to the wells. Predetermined concentrations of monoclonal antibody purified by affinity chromatography were run in parallel in the same plate and used as a reference standard. After overnight incubation at room temperature, the plates were washed again and developed wth 50 λ of 50,000 cpm/50 λ ^{125}I-labeled goat anti-mouse Ig. The wells were finally washed 15 times and counted in a Beckman γ-counter. The concentration of each monoclonal antibody in the ascites fluid was calculated according to the standard curve obtained with its respective purified monoclonal antibody.

Iodination of Purified Antibodies

Affinity-purified antisera or monoclonal antibodies were iodinated with $Na^{125}I$ (low pH, carrier-free $Na^{125}I$, from New England Nuclear, Boston, MA) according to the Chloramine T method described by Greenwood, et al. (1963). The non-protein-bound radioactive iodine and small molecular weight reagents were removed on a G-50 column by centrifuging the reaction mixture containing 1 ml G-50 sepharose for 2 minutes at 900 rpm in a Beckman model TJ-6 Centrifuge (Palo Alto, CA) (Penefsky and Harvey, 1977).

Studies of the Biological Activity of Monoclonal Antibodies in Mice

The biological activity of monoclonal antibodies XY101 and XY103 (both are X31 HA specific) in mice infected with X31 virus were studied in assays equivalent to the pre-inoculation neutralization and post-inoculation plaque reduction assays in tissue culture.

(i) The effect of virus infection in mice preinoculated with monoclonal antibodies (equivalent to pre- and post-inoculation neutralization assay in tissue culture):

CD-1 pathogen-free, male mice were first injected intraperitoneally with 1:15 dilutions of ascites fluids of XY101 or XY103. Control mice were injected with PBS. Twenty-four hours later, serial 5-fold dilutions ranging from 10^{-2} to $10^{-6.2}$ of X31 allantoic fluid seed virus were used to infect the mice by aerosol (8 mice/virus dilution). The apparatus and technique of aerosol infection was performed according to a procedure described by Schulman and Kilbourne (1963a).

Mice were exanginated and lungs were removed aseptically 3 days later. The presence of virus in the lung was determined by a method described by Schulman and Kilbourne (1963b). Briefly, lungs in ABS-gel

(PBS containing 500 U/ml penicillin, 5 mg/ml streptomycin and 0.1% gelatin) were ground with telflon grinders at 4°C, and the suspensions were centrifuged at 2,500 rpm for 15 minutes in a Beckman model TJ-6 centrituge (Palo Alto, CA). The supernatants were diluted to 1:100 in PBS containing 500 U/ml penicillin and 5 mg/ml streptomycin and 0.1 ml were inoculated into the allantoic cavity of 10 to 11-day old chick embryos. After 40 to 48 hours of incubation, viral hemagglutinin was tested at a 1:4 dilution in macrowells (Becton Dickinson Co., Oxnard, CA) with 0.5% human 'O' type RBC. The dilution of virus at which 50% of the mice were infected was expressed as the mouse infectious dose$_{50}$ (MID$_{50}$) according to a standard method (Schulman and Kilbourne, 1963a).

(ii) The effect of monoclonal antibodies in mice preinfected with X31 virus (equivalent to the postinoculation plaque reduction assay in tissue culture):

Mice were exposed to aerosol infection using a 1:10 dilution of X31 allantoic seed virus according to a standard procedure established in this laboratory (Schulman and kilbourne, 1963a). Four hours after infection, the mice were inoculated with concentrations of XY101 or XY103 containing equivalent HI activity or with PBS intraperitoneally. Lungs were removed aseptically 3 and 7 days later, and pulmonary lesions were scored on day 7 (Horsfall, 1939).

The titers of virus in lungs were determined according to the method described by Schulman and Kilbourne (1963a). Briefly, 0.1 ml of various 10-fold dilutions of supernatants of ground lung suspensions were inoculated into chick embryo in triplicate. The presence of viral HA activity was detected in macrowells after 40 to 48 hours incubation at

37°C. The virus titers in lungs were expressed as EID_{50} (egg infectious $dose_{50}$; the dilution of supernatants infecting 50% of embryonated eggs).

Preparation of Anti-idiotype Antisera

Syngeneic anti-idiotype antisera against monoclonal antibodies B118, B123, B142 and B147 (specific for B/Lee HA) were raised in BALB/c mice using a method described by Bona, et al. (1979). Monoclonal antibodies purified on BLee-sepharose column were coupled to keyhole limpet hemocyanin (KLH) (1:1) by glutaraldehyde (Bona, et al., 1979). BALB/c mice were immunized with 75 µg of monoclonal antibody-KLH emulsified in complete Freund's adjuvant (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) then 5 days later with 75 µg of monoclonal antibody-KLH in incomplete Freund's adjuvant followed by 75 µg of monoclonal antibody-KLH in saline weekly for 6 weeks. After the last immunization, mice were bled at intervals for two months and sera of individual mice were pooled.

Homologous anti-idiotype antisera against monoclonal antibody P20 and P28 (specific for PR8 HA) were prepared by similar procedures except that monoclonal antibodies were not coupled to KLH before injection and A/J mice were used instead of BALB/c mice. All of the homologous antisera were adsorbed on a BALB/c Ig column to eliminate anti-allotype antibodies before further purification. (Initial efforts to raise anti-P20 and anti-P28 antibody in BALB/c mice were unsuccessful).

Purification of Anti-idiotype Antibodies by Affinity Column Chromatography

The anti-idiotype antisera were purified on corresponding monoclonal antibody-sepharose columns using methods similar to those employed in

purifying monoclonal antibodies over virus-sepharose columns. Monoclonal antibody-sepharose columns were made using monoclonal antibodies purified in two steps:

Step One: Precipitation with Ammonium Sulfate

One volume of saturated ammonium sulfate (Schwartz-Mann, Orangeburg, NY) was added dropwise to one volume of ascites fluid containing monoclonal antibodies. The suspensions were stirred for at least 10 minutes to reach equilibrium, and precipitates were sedimented by centrifugation at 10,000 rpm for one hour in a Sorvall Centrifuge (Sorvall Instruments, Newton, CT). The precipitates then were dissolved in a minimum volume of PBS and the solutions then were dialyzed against 4 liters of 40 mM phosphate buffer (pH 8.0) overnight at 4°C.

Step Two: DEAE Chromatography

Diethylaminoethyl cellulose (DE52) (Whatman Chemical, England) columns were packed according to the instructions provided by the manufacturer and equilibrated with 10 column volume of 40 mM phosphate buffer (pH 8.0). The nondialyzable material from step 1 was loaded onto the column and in a single step eluted with 40 mM phosphate buffer. The protein containing fractions were detected at absorbance of 280 nm. Positive fractiosn were pooled, concentrated with Carbowax (PEG 20,000, Fisher, Fairlawn, NJ) and dialyzed overnight against 0.9% NaCl.

Assays for Idiotypes

The assays for cross-reactive idiotypes included HI and RIA, and were performed as follows:

(i) Hemagglutination inhibition assay (HI). Sheep red blood cells (SRBC) were coated with affinity purified monoclonal antibodies using the chromic chloride ($CrCl_3$) method (Bona, et al., 1978) as follows: 0.1 ml

affinity purified monoclonal antibodies (at concentrations of 0.7 to 1.0 mg/ml), 0.1 ml 0.1% $CrCl_3$ in saline and 0.1 ml 50% SRBC were mixed and reacted in a small test tube. The reaction was stopped with about 20 ml 0.9% saline as soon as the SRBC started to agglutinate (approximately 3 minutes). The suspension of SRBC coated with monoclonal antibodies then was washed 3 times with saline and resuspended in 5 ml slaine (3% FCS). Syngeneic or homologous anti-idiotype sera then were assayed to determine the dilution at which hemagglutination of coated SRBC was still present. In these assays a positive control was included in the form of rabbit anti-mouse I_g sera to verify that coupling of monoclonal antibodies to the SRBC had occured. Negative controls included saline to ensure that spontaneous agglutination of SRBC was not observed. Anti-idiotype serum was diluted to a concentration at which it still had a hemagglutination titer of 1:3 (i.e. agglutination was observed only in the first well). Then, the capacity of various monoclonal antibodies to inhibit agglutination of these coated erythrocytes by anti-idiotype antisera were determined according to the methods described by Bona, et al. (1978). The HI titers were reported as $\log_2$ of the reciprocal of the highest dilution of inhibitors which inhibited hemagglutination.

(ii) RIA (Bona, et al., 1980a). RIA of both direct binding and competition inhibition were used.

(a) Direct RIA. Microtiter wells were coated with concentrations of purified anti-idiotype antibodies ranging from 1 μg/ml to 100 μg/ml overnight at 4°C. After 3 washings, they were incubated for 90 minutes at room temperature with 50% FCS, and then with various ^{125}I-labeled monoclonal antibodies (50,000 cpm) for 3 hours. After extensive washing, the wells were cut and counted in the γ-counter.

(b) Competition Inhibition RIA. In preliminary assays, different concentrations of purified anti-idiotype antibody were used to coat microtiter wells and the binding of a constant amount (50,000 cpm) of purified ^{125}I-labeled monoclonal antibody was measured. Concentrations of the purified anti-idiotype antibody which bound the monoclonal antibody within the linear range then were used in the inhibition assay. In this assay, various concentrations of different unlabeled purified monoclonal antibodies were tested with respect to their ability to inhibit the binding of anti-id antibodies with homologous or heterologous ^{125}I-labeled monoclonal antibodies.

Viral Inhibition Assay

To determine the ability of purified virus to inhibit the binding of monoclonal antibodies with anti-id antibodies, mixtures of ^{125}I-labeled monoclonal antibodies and various concentrations of purified virus were preincubated for one hour, and then added to plates coated with anti-id antibodies. The concentration of purified virus that caused a 50% reduction of binding of ^{125}I-monoclonal antibodies to anti-id antibody-coated wells was determined.

Determination of Protein Concentration

The protein concentration of purified monoclonal antibodies and viruses was determined either by Lowry assay (Lowry, et al., 1951) or by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Immunization and Bleeding

(i) To study the idiotype of anti-HA antibodies in young mice.

One, 7, 14, 21, and 28 day old BALB/c mice were injected with 10 μg/mouse of purified BLee virus intraperitoneally. The mice were bled 5 days after immunization. The titer of anti-viral hemagglutinating inhibition antibody was determined by standard HI assay, and the expression of a cross-reactive Id shared with monoclonal antibody was studied in HI assay or by RIA.

(ii) To study the idiotypic expression during primary and secondary anti-HA antibody responses in adult BLAB/c mice.

Twenty-four adult BALB/c mice were divided into two groups. In the primary immune response group, mice were immunized with 10 μg/mouse of purified B/Lee virus and bled 5, 10, 20, 30 and 60 days after immunization. In the secondary immune response group, mice primed with 10 μg/mouse of purified B/Lee virus were injected with the same inoculum 4 weeks later, and then bled 3, 7, 20 and 30 days after boost. Anti-viral HA titers of antisera pooled from pairs of mice in the group of 12 mice were measured in anti-viral HI assays and radioimmunoassay.

(iii) To study the genetics of anti-HA antibody response.

The expression of idiotypes of monoclonal antibodies specific for B/Lee virus HA in various strains of inbred mice were studied in antisera obtained after primary and secondary immunization with B/Lee virus. The strains of mice included in the study were BALB/c, BALB.B, BALB.K, BAB.14, C.B20, C.AL20, DBA/1J, DBA/2J, CBA/J, PL/J, A/J, AKR/J, SWR/J, RIIIS/J, C57BL/6, CE/J and C3H/J. All mice were immunized with purified B/Lee virus (10 μg/mouse) and then bled 10 and 20 days after immunization. The mice were reimmunized with the same concentration of

virus one month after priming, and bled 7 and 20 days later. The anti-viral HA titers of the sera were measured in HI assay. The expression of idiotypes shared with monoclonal antibodies was studied by RIA.

(iv) To study the idiotypic expression of BALB/c mice immunized with natural variants of B/Lee virus.

Adult BALB/c mice were immunized intraperitoneally with purified B/Great Lake/54, B/Hong Kong/8/72, B/Victoria/70 or B/Maryland/59 virus (10 μg protein/muse) and reimmunized one month later with the same amount of the same virus. Sera were obtained 10 days after primary immunization and 7 days after secondary immunization. The HI titers against the immunizing viruses and against B/Lee virus were determined, and the expression of idiotypes shared with B/Lee monoclonal antibodies were studied by RIA.

(v) To study the idiotypic expression of various species of animals immunized with B/Lee virus.

Immunization and bleeding of chickens, guinea pigs, rats and rabbits were performed by Pocono Rabbit Farm and Laboratory (Canadensis, PA) as follows: All animals were immunized intravenously with 100 μg protein/animal of purified B/Lee virus and bled 10 days later. These animals were reimmunized with the same amount of virus on day 40 and exaguinated on day 47. Human cord blood serum was a gift from Dr. F. Siegal (Department of Medicine, Mt. Sinai Medical Center, NY). The adult human sera were collected from volunteer donors within the laboratory. The anti-B/Lee HI titers of these sera were studied, and the expression of cross-reactive idiotypes shared by B/Lee monoclonal antibodies was determined by competition inhibition RIA.

VIII. RESULTS

A. CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO INFLUENZA VIRUSES IN TERMS OF ANTIVIRAL HI TITERS, SUBCLASSES AND FINE SPECIFICITIES

(i) Monoclonal antibodies specific for PR8 virus

The monoclonal antibodies to PR8 virus HA used in these studies were P8, P20 and P28. Their antiviral HI titers and subclasses are listed in Table II. All three monoclonal antibodies have similar antiviral HI activity, and P8, P20 and P28 belong to subclasses IgG_{2b}, IgG_{2b}, and IgG_1, respectively.

These monoclonal antibodies were determined to be specific for distinct antigenic determinants on PR8 HA on the basis of the following criteria: (a) the frequencies of antigenic variants in the presence of pairs of monoclonal antibodies were equivalent to the products of those frequencies observed in the presence of each monoclonal antibody alone (Lubeck, et al., 1980); (b) when variants selected in the presence of a particular monoclonal antibody were examined in HI or neutralization assays monoclonal antibodies directed to other determinants still reacted at unchanged titer. The HI titers of P8, P20 and P28 to PR8 virus and variants are shown in Table III. It can be seen that variants selected in the presence of P8 (P8 variants), in the presence of P20 (P20 variants) or in the presence of P28 (P28 variants) did not react with the monoclonal antibodies used in selection. However, the titers of monoclonal antibodies not used in the selection remained relatively unchanged within the range of error of the HI assay. For instance, P8 no longer reacted with P8 variants but P20 and P28 still reacted with P8 variants at high titers. Thus, it is evident that P8, P20 and P28 are reactive with different determinants on the PR8 HA molecule.

Table II

SUMMARY OF ANTI-VIRAL HEMAGGLUTINATION INHIBITION (HI) TITERS

AND SUBCLASSES OF MONOCLONAL ANTIBODIES AGAINST PR8 VIRUS

Monoclonal antibodies	HI titers* (log_2 units)	Subclasses
P8	7	Ig G_{2b}
P20	8	Ig G_{2b}
P28	8	Ig G_1

*Two-fold dilutions of RDE treated ascites were used to inhibit the hemagglutination of human RBC by 16-32 hemagglutinating units of PR8 virus. The titers are expressed as the reciprocal (log_2) of the highest dilution of RDE treated ascites that causes inhibition of hemagglutination.

Table III

REACTIVITY OF MONOCLONAL ANTIBODIES TO PR8 VIRUS HA WITH VARIANTS SELECTED WITH DIFFERENT MONOCLONAL ANTIBODIES

Viruses	Number of variants examined	Range of HI titers (log_2 units)† P8	P20	P28
PR8	-	7	8	8
P8 Variants*	8	< 1	6∿7Δ	6∿8
P20 Variants*	7	5∿7	< 1	6∿8
P28 Variants*	6	5∿7	6∿7	≤ 1

*P8, P20 and P28 variants were variants of PR8 virus selected in the presence of excess P8, P20 and P28 monoclonal antibodies, respectively.

†Reciprocal of dilution of monoclonal antibody (log_2) which inhibits agglutination of human "O" erythrocytes by 3-4 agglutinating units of test virus.

ΔRange of HI titers of each monoclonal antibody with different variants selected in the presence of a single monoclonal antibody.

(ii) Monoclonal antibodies specific for X31 virus

The monoclonal antibodies to X31 virus HA used in these studies were XY101, XY102, XY103, XY104, XY105, XY106, XY107, XY108, X146, X188 and X202. Their antiviral HI titers and subclasses are listed in Table IV. All X31 monoclonal antibodies have similar antiviral HI activity. XY103, XY104, XY105, XY106 and XY107 belong to subclass IgG_1; XY101, XY108, X146 and X188 belong to subclass IgG_{2a}; and XY102 belongs to subclass IgG_{2b}.

Specificities of these monoclonal antibodies were determined according to HI results of these monoclonal antibodies with a series of antigenic variants. As seen in Table V, it was difficult to precisely classify the monoclonal antibodies to X31 HA into different groups with respect to the epitopes to which they are directed. XY101 does not react with variants selected by XY104, nor does XY104 react with variants selected by XY101. In addition, these two monoclonal antibodies have the same pattern of reactivity with variants selected by the other monoclonal antibodies. Thus XY101 and XY104 appear to be directed to identical or closely overlapping determinants. However, classification of the other monoclonal antibodies is difficult because of two observations: (a) asymmetry of reactivity; (b) differences in reactivity with different variants selected by a single monoclonal antibody. The first of these is illustrated by XY 107 and X146. XY 107 does not react with variants selected by X146 but X146 has the same or only slightly reduced reactivity with variants selected by XY107. Similar asymmetry can be seen with XY 103, XY 101 and XY 104 in that XY 103 shows reduced reactivity with variants selected by XY101 or XY104 but the latter two monoclonal antibodies do not have reduced HI titers against variants selected by XY103. The second observation is illustrated by monoclonal

TABLE IV

SUMMARY OF ANTI-VIRAL HEMAGGLUTINATION INHIBITION (HI) TITERS

AND SUBCLASSES OF MONOCLONAL ANTIBODIES AGAINST X31 VIRUS

Monoclonal antibodies	HI titers (log_2 units)	Subclasses
XY101	8∿11	Ig G_{2a}
XY102	7∿11	Ig G_{2b}
XY103	8∿12	Ig G_1
XY104	9∿13	Ig G_1
XY105	8∿12	Ig G_1
XY106	10∿14	Ig G_1
XY107	9∿10	Ig G_1
XY108	4∿11	Ig G_{2a}
X146	6∿9	Ig G_{2a}
X188	8∿14	Ig G_{2a}
X202	9∿15	ND

ND = Not determined

*Two-fold dilutions of RDE treated ascites were used to inhibit the hemagglutination of human RBC by 16-32 hemagglutinating units of X31 virus. The titers are expressed as the reciprocal (log_2) of the highest dilution that causes inhibition of hemagglutination.

TABLE V

REACTIVITY OF MONOCLONAL ANTIBODIES TO X31 VARIANTS SELECTED IN THE PRESENCE OF VARIOUS MONOCLONAL ANTIBODIES

Virus	Number of variants examined	Ratio of HI titers ($\log_2$ units)‡ $\left(\frac{\text{wild type}}{\text{variants}}\right)$										
		XY103	XY101	XY104	XY105	XY106	XY108	XY102	XY107	X146	X188	X202
XY103 variants*	3	8	0-1	0	0-1	0	0-3	0-5	0-1	0	0-5	0-1
XY101 variants*	3	4-8	10∿11	9∿13	9∿12	9∿13	4∿8	0-1	0	0∿4	0∿6	0∿6
XY104 variants*	4	5∿6	9	9	9	8∿10	6∿8	0-1	0	2	0∿2	0∿1
XY105 variants*	4	4∿8	3∿10	0∿13	7∿12	1∿12	0∿8	0∿1	0∿1	0∿5	0∿6	0∿4
XY106 variants*	4	4∿6	1∿9	3∿10	2∿9	8∿10	1∿10	1∿5	0∿7	1∿5	0∿1	0∿13
XY108 variants*	4	4∿5	1∿10	3∿11	3∿9	6∿10	8∿10	0∿1	0∿2	1∿2	0∿8	0∿4
XY102 variants*	2	0∿5	0∿2	0∿1	0∿1	1∿6	0∿1	4∿6	0∿1	0∿6	0∿6	0∿1
XY107 variants*	3	0∿1	0	0∿2	0	0	0	0∿1	9∿10	0∿1	0∿1	0
X146 variants*	4	0∿7	0∿7	0∿10	1∿8	10∿14	0∿9	0∿6	8∿10	7∿8	0∿3	0∿2
X188 variants*	3	0∿2	0	0	0	0∿1	0∿1	0∿1	0	0	8∿10	6∿8
X202 variants*	3	0∿7	0∿3	0	0∿3	0∿1	0∿1	0∿1	0∿1	0∿3	0∿13	9∿10

*XY103, XY101, XY104, XY105, XY106, XY108, X202, X188 and X202 variants are selected in the presence of XY103, XY104, XY105, XY106, XY108, X188 and X202 monoclonal antibodies, respectively.

‡Ratio of HI titers measured by RDE treated monoclonal antibody versus wild type X31 virus and versus variants. For example, if the HI titer of a monoclonal antibody with X31 virus is 12($\log_2$) and its titer with a variant is 4($\log_2$) the ratio is expressed as 8. If the ratio is given as 0, the monoclonal antibody reacts with all variants at unchanged titer.

antibodies X146 and XY105. Monoclonal antibody X146 reacts with some but not all variants selected by XY105 at significantly reduced titer. Asymmetry of reactivity with variants can be attributed either to differences in the avidity of different monoclonal antibodies and/or to the possibility that some mutations in or near one epitope may through conformational changes affect the binding of monoclonal antibodies directed to another epitope. The finding of differences in reactivity of a monoclonal antibody with different variants selected by another monoclonal antibody could be due either to the possibility that the two monoclonal antibodies recognize overlapping determinants or again to conformational changes affecting non-overlapping epitopes.

The frequencies of antigenic variants detected in cloned X31 virus seeds in the presence of a single monoclonal antibody and of two combined monoclonal antibodies specific for X31 were also determined to evaluate whether these monoclonal antibodies are against the same antigenic area. From the results shown in Table VI, it can be seen that XY101, XY104, XY105, X146, X188 and X202 are against overlapping antigenic regions, whereas XY102 and XY107 are against distinct antigenic determinants. Thus, when either XY102 or XY107 was combined with each other or with any other monoclonal antibody no variants were detected indicating that more than a single mutation would be required for a variant to be resistant to both antibodies. In contrast, combinations of any 2 of the other monoclonal antibodies did not affect the detected frequency of variation compared to that observed when either monoclonal antibody was employed alone.

TABLE VI

FREQUENCY OF ANTIGENIC VARIANTS DETECTED IN A CLONED INFLUENZA X31 VIRUS SEED IN THE PRESENCE OF A SINGLE MONOCLONAL ANTIBODY OR COMBINED MONOCLONAL ANTIBODIES

Monoclonal antibody	Detected frequency of antigenic variants(log_{10})[§]
XY101	-6.7
XY102	-6.4
XY103	-5.8
XY104	-6.9
XY105	-6.1
XY106	-7.1
XY107	-6.7
XY108	-6.6
X146	-7.4
X188	-6.1
X202	-6.2
XY101 + XY104	-7.0
XY104 + XY105	-7.0
XY104 + XY146	-7.7
X188 + X202	-6.2
XY102 + A*	<-7.7
XY107 + B**	<-7.7
XY101 + X202	-7.4

*A= monoclonal antibody XY101, XY104, XY105, XY106, XY107, X146, X188 or X202.

**B= monoclonal antibody XY101, XY102, XY104, XY105, X146, X188 or X202

§ PFU/ml (variants)/PFU/ml (total)

(iii) Monoclonal antibodies specific for BLee virus

The HI titers and subclasses of the monoclonal antibodies to B/Lee HA are listed in Table VII. Again, these monoclonal antibodies have similar HI titers, and B109, B118, B123, B127 and B141 belong to subclass IgG_1, but B142 belongs to subclass IgG_{2a}.

These monoclonal antibodies were determined to be directed against the same or overlapping epitopes in part on the basis of HI results shown in Table VIII. In all cases, monoclonal antibodies which were used to select variants either did not react with those variants or reacted at significantly lower titer than with wild type virus. In addition, as summarized in Table VIII, monoclonal antibodies other than those used to select a particular group of variants did not react or reacted at significantly lower titer with at least some of the variants in that group than with wild type virus. For example, all of the monoclonal antibodies tested reacted at lower titer with all six variants selected in the presence of B118 than with wild type virus. Similar results were obtained in HI assays with at least some of the variants selected with other individual monoclonal antibodies. In addition, when these variants were tested in neutralization assay with the monoclonal antibodies listed in Table VII, reduced reactivity was observed in all instances. Finally, the frequency of antigenic variants detected in cloned virus seeds in the presence of a single monoclonal antibody was equivalent to that observed when any two monoclonal antibodies were combined (Table IX).

The localization of antigenic determinant to which monoclonal antibody B147 is directed is more complicated due to the following observation: Although B147 reacted at reduced titer with all but a few of the variants selected in the presence of other monoclonal antibodies,

TABLE VII

ANTI-VIRAL HEMAGGLUTINATION INHIBITION (HI) TITERS

AND SUBCLASSES OF MONOCLONAL ANTIBODIES AGAINST B/LEE VIRUS

Monoclonal antibodies	HI titers* (log_2 units)	Subclasses
B109	7	Ig G_1
B118	8	Ig G_1
B123	9	Ig G_1
B127	8	Ig G_1
B141	8	Ig G_1
B142	7	Ig G_{2a}
B147	9	ND

*Two-fold dilutions of RDE treated ascites were used to inhibit the hemagglutination of human RBC by 16-32 hemagglutinating units of B/Lee virus. The titers are expressed as the reciprocal (log_2) of the highest dilution that causes inhibition of hemagglutination.

ND - Not determined.

TABLE VIII

REACTIVITY OF MONOCLONAL ANTIBODIES TO B/LEE VIRUS HA WITH VARIANTS SELECTED WITH DIFFERENT MONOCLONAL ANTIBODIES

Viruses	Number of variants examined	Range of HI titers (log_2 units)† B109	B127	B142	B141	B123	B118	B147
B/Lee	-	7	8	7	8	9	8	9
B109 variants*	6	<1	<1	<1	4~9Δ	4~7	5~8	1~9
B127 variants*	6	<1	<1	<1	<1~5	5	<1~7	<1~6
B142 variants*	4	<1	<1	<1	<1~9	4~9	<1~7	2~6
B141 variants*	6	<1	<1	<1	2~6	5~7	3~6	<1~8
B123 variants*	2	<1	<1~4	<1	4~6	<1~3	4	< 1
B118 variants*	6	<1~4	<1~6	4~6	<1~6	4~6	<1~4	< 1
B147 variants*	2	6~7	5~6	6~7	7~8	5~6	5~6	< 1

*B109, B127, B142, B141, B123, B118 and B147 variants were variants of B/Lee viruses selected in the presence of excess B109, B127, B142, B141, B123, B118 and B147 monoclonal antibodies, respectively.

†Same as table II, except that either B/Lee virus or variants were used.

ΔRange of HI titers of each monoclonal antibody with different variants selected in the presence of a single monoclonal antibody.

TABLE IX

FREQUENCY OF ANTIGENIC VARIANTS DETECTED IN A CLONED INFLUENZA B/LEE VIRUS SEED IN THE PRESENCE OF A SINGLE MONOCLONAL ANTIBODY OR COMBINED MONOCLONAL ANTIBODIES

Monoclonal antibody	Detected frequency of antigenic variants (log_{10})*
B109	-6.0
B118	-7.7
B123	-7.0
B127	-5.8
B141	-7.1
B142	-6.1
B147	-6.0
B109 + B118	-6.8
B118 + B123	-7.1
B109 + B127	-6.1
B109 + B142	-6.1
B118 + B141	-7.1
B127 + B142	-6.2
B141 + B142	-7.2
B118 + B147	-7.2

*PFU/ml(variants) / PFU/ml(total)

other monoclonal antibodies reacted with the two variants selected in the presence of B147 at relatively high titer. This assymmetry suggests that B147 is either directed at a determinant which overlaps with that recognized by the other monoclonal antibodies or that mutations in or near the antigenic area recognized by other monoclonal antibodies via conformational changes affect the binding of monoclonal antibody directed to epitope recognized by B147. To differentiate between these two possibilities, frequencies detected in the presence of B147 and one of the other monoclonal antibodies were determined. As shown in Table IX, the frequency of variants detected in the presence of both B118 and B142 is equivalent to these detected in the presence of a single monoclonal antibody. This observation supports the first possibility that B147 is directed at a determinant which overlaps with that recognized by other monoclonal antibodies.

B. FACTORS WHICH MAY INFLUENCE THE DETECTED FREQUENCY OF ANTIGENIC VARIANTS

I. Detection of frequency of antigenic variants using undiluted RDE-treated ascites (i.e. at antibody excess)

The monoclonal antibodies to BLee, PR8 and X31 virus HAs were used in the study. Comparisons of detected frequencies of antigenic variants, binding constants of monoclonal antibodies, concentrations of monoclonal antibodies in ascites fluids and isotypes were carried out with: (a) different monoclonal antibodies against the same or overlapping antigenic determinant(s) in the BLee virus system, (b) a single monoclonal antibody specific for BLee or X31 virus HAs and different variants of these viruses, and (c) monoclonal antibodies recognizing different antigenic determinants on PR8 and X31 viruses.

(i) Monoclonal antibodies specific for BLee virus

B109, B118, B123, B141 and B142 were determined to be monoclonal antibodies against the same (or overlapping) antigenic determinant(s) on HA of BLee virus as discussed previously. As seen in Table X, the frequencies of antigenic variants detected by these monoclonal antibodies varied from $10^{-5.5}$ to $<10^{-8.1}$. These differences were correlated with differences in the binding constants of the monoclonal antibodies examined. In other words, there was an inverse relationship between antibody avidity and detected frequency of antigenic variants. Neither isotope or differences in concentration of monoclonal antibody employed seemed to affect the detected frequencies of antigenic variants in this system.

This inverse relationship between antibody avidity and detected frequency of antigenic variants was illustrated more clearly when we used a single monoclonal antibody (B118) to compare the avidity, preinoculation neutralization titer and detected frequency of antigenic variants with respect to B/Lee virus and two variants of BLee virus, B109m11 (a variant of BLee selected in the presence of B109) and B/MD/59 (a natural variant) (Table XI). In this system, the antibody concentration and the antigenic site involved were the same since only one monoclonal antibody was employed. A similar inverse relationship between antibody avidity and detected frequency of variants was again observed. Furthermore, this inverse relationship was also observed between detected frequency of variants and preinoculation neutralization titers. The latter observation can simply be explained by the fact that when antibody concentration was a constant, differences in antibody avidity were clearly related to differences in preinoculation neutralization titer.

TABLE X

RELATIONSHIP OF AVIDITY AND DETECTED FREQUENCY OF ANTIGENIC VARIANTS FOR MONOCLONAL ANTIBODIES AGAINST THE SAME ANTIGENIC AREA IN B/LEE/40 HEMAGGLUTININ

Monoclonal antibody	Concentration in ascites(mg/ml)	Binding constant (K) M^{-1})	Detected frequency(log_{10}) of antigenic variants*
B109	3.3	-2.0×10^9	-5.5 ± 0.5
B127	12.7	-1.4×10^9	-5.6 ± 0.5
B142	3.6	-1.8×10^9	-5.6 ± 0.5
B141	9.5	-4.6×10^9	-6.9 ± 0.3
B118	9.6	-6.8×10^9	$<-8.1 \pm 0.4$

*The frequencies were determined using 4 cloned virus seeds, and are given as mean $\pm$ S.D. in log_{10} units.

TABLE XI

RELATIONSHIP OF DETECTED FREQUENCY OF ANTIGENIC VARIANTS, PREINOCULATION NAUTRALIZATION TITER, AND BINDING CONSTANT(K) OF MONOCLONAL ANTIBODY B118 VERSUS B/LEE/40, B109m11 and B/MD/59 VIRUSES

Virus	Detected frequency of antigenic variants (log_{10})	Preinoculation neutralization titer	Binding constant(K) (M^{-1})
B/Lee/40	$<-8.1 \pm 0.4$	370,000	-6.8×10^9
B109m11	-6.0 ± 0.5	24,000	-1.8×10^9
B/Md/59	-5.2 ± 0.5	4,000	-1.0×10^9

*The data with B/Lee/40, B109m11, and B/Md/59 viruses were calculated from results of 4,2 and 5 cloned seeds, respectively.

(ii) Monoclonal antibodies specific for PR8 virus

P8, P20, and P28 are monoclonal antibodies against different antigenic determinants on PR8 HAs as mentioned section (Ai). When these monoclonal antibodies were studied with respect to factors that might influence the detected frequency of antigenic variants (Table XII), we did not observe a clear relationship between antibody avidity and detected frequency of variants. Again, the concentration of monoclonal antibody in ascites fluids and subclasses did not have an effect on detected frequency of antigenic variants. Thus, it is evident that factors other than antibody avidity, antibody concentration and subclasses have an effect on detected frequencies of antigenic variants.

(iii) Monoclonal antibodies specific for X31 virus

To gain additional information about the factors that might influence the detected frequency of antigenic variants in cloned virus seeds, a panel of 11 monoclonal antibodies to X31 virus were included in a further study. XY101 and XY104 are against the same (or very closely located) determinant(s) on X31 HA (as mentioned in section Aii). As shown in Table XIII, the detected frequencies of antigenic variants in the presence of these 2 monoclonal antibodies were similar, as were their binding constants. But when the results of other 9 monoclonal antibodies were compared, the factors that influence the detected frequency of antigenic variants were not clear. It is true that X146 has the highest binding constant and the lowest detected frequency of variants, but it is evident that this correlation does not hold up when other monoclonal antibodies are considered. For example, XY102 and XY105 have equivalent binding constants but there is more than a 500 fold difference in the frequencies of antigenic variants detected with these two monoclonal

antibodies. It should be noted that the concentration of antibody in XY102 ascites was more than 2 fold greater than that in XY105 ascites but from the data in Table XIII it is evident that antibody concentration alone is not the determining factor as evidenced by the fact that although XY105 ascites contained approximately 8 fold higher antibody concentration than XY108, the detected frequencies of antigenic variants with these two monoclonal antibodies were almost identical. The effects of antibody concentration will be described in more detail in another section. Hence, from the studies of X31 monoclonal antibodies, it was clear that factors other than subclass, antibody concentration and antibody avidity have an effect on the detected frequency of antigenic variants.

In other experiments, the relationship of antibody avidity to preinoculation neutralization titer and to detected frequency of antigenic variants was examined using a single monoclonal antibody, X146, in assays with X31 virus and three laboratory variants of X31 virus selected in the presence of other monoclonal antibodies; XY106m4, XY105m5 and XY108m5. As seen in Table XIV, the detected frequencies of variants with XY106m4, XY105m5 were higher than with X31. In addition, the preinoculation neutralization titers and binding constants of X146 with these variants were lower. Thus, these results with a single X31 virus specific monoclonal antibody and different virus variants are similar to those observed in the B/Lee virus system and demonstrate an inverse relationship between antibody avidity and detected frequency of variants.

TABLE XII

RELATIONSHIP OF BINDING CONSTANT AND FREQUENCY OF DETECTED ANTIGENIC VARIANTS FOR MONOCLONAL ANTIBODIES AGAINST DIFFERENT DETERMINANTS OF PR8 VIRUS HEMAGGLUTININ

Monoclonal antibody	Concentration in ascites(mg/ml)	Binding constant (K) (M^{-1})	Detected frequency (log_{10}) of antigenic variants*
P8	10.5	-1.0×10^9	-4.4 ± 0.5
P28	10.1	-1.9×10^9	-6.2 ± 0.6
P20	14.1	-4.4×10^9	-7.0 ± 0.5

*The frequencies were results of 4 cloned virus seeds, and are expressed as means $\pm$ S.D. in log_{10} units

TABLE XIII

RELATIONSHIP OF AVIDITY AND FREQUENCY OF DETECTED ANTIGENIC VARIANTS FOR MONOCLONAL ANTIBODIES AGAINST X31 HEMAGGLUTININ

Monoclonal antibodies	Concentration in ascites (mg/ml)	Binding constant (K) (M^{-1})	Detected frequency of antigenic variants(log_{10})*
X146	2.2	-5.3×10^9	$<7.4 \pm 0.1$
XY102	9.5	-1.6×10^9	$<7.1 \pm 0.5$
XY103	7.7	-2.7×10^9	-6.8 ± 0.7
XY104	4.5	-1.3×10^9	-5.8 ± 0.3
X188	5.3	-1.6×10^9	-5.6 ± 0.8
XY101	2.9	-1.8×10^9	-5.4 ± 1.0
XY106	6.5	$-1.8 + 10^9$	-5.2 ± 0.6
X202	6.9	$-4.5 + 10^8$	-5.2 ± 0.7
XY107	3.6	$-1.8 + 10^9$	-4.6 ± 0.8
XY108	0.58	$-1.4 + 10^9$	-4.6 ± 0.4
XY105	4.8	-1.7×10^9	-4.4 ± 0.4

*The frequencies were determined using 3 cloned virus seeds, and are expressed as mean $\pm$ S.D. in log_{10} units.

TABLE XIV

RELATIONSHIP OF DETECTED FREQUENCY OF ANTIGENIC VARIANTS, PREINOCULATION NEUTRALIZATION TITER, AND BINDING CONSTANT(K) OF MONOCLONAL ANTIBODY X146 VERSUS X31 VIRUS AND LABORATORY VARIANTS

Virus	Detect Frequency of antigenic variants(log_{10})	Preinoculation neutralization titer	Binding constant (K) (M^{-1})*
X31P3	-7.4	22,000	-5.3×10^9
XY108m5§	-4.7	4,500	-3.1×10^9
XY105m5§	-3.4	2,300	-2.6×10^9
XY106m4§	-3.9	1,500	-2.2×10^9

*The 4 binding constants reported here were detected at the same time.

§XY108m5, XY105m5 and XY106m4 are variants selected in the presence of XY108, XY105 and XY106 monoclonal antibodies, respectively.

II. Relationship between concentration of monoclonal antibody and frequency of detected variants

To assess the effects of antibody concentration on the frequency of detected variants, serial four-fold dilutions of RDE-treated ascites fluids containing monoclonal antibodies were employed to assay the number of detected variants. In this study, plaques picked at all antibody concentrations were screened carefully by both HI and neutralization test with homologous monoclonal antibodies to ensure that all surviving virus populations were variants. With the monoclonal antibodies we employed in these studies, two patterns were observed. With the majority of monoclonal antibodies, as the concentration of antibody was reduced, the frequency of detected variants was relatively unchanged until a concentration was reached at which significant numbers of un-neutralized wild type virus survived (e.g. XY101, XY107,XY108, XY104, Table XV). However, for certain monoclonal antibodies, such as XY106 and particularly XY102 (as shown in Table XV), the detected frequency of variants increased as the concentration of the monoclonl antibody decreased within a certain range. When the antibody concentration was reduced below this range, the frequency of antigenic variants could not be determined, again because of the presence of wild type virus in the surviving virus population.

III. Detected frequencies of antigenic variants in different assay systems

In another attempt to elucidate factors that might contribute to the differences in frequency of detected variants, different assay systems were studied. In one assay system, monoclonal antibodies were present both before and after virus adsorption (as the method employed in the

TABLE XV

EFFECT OF ANTIBODY CONCENTRATION ON DETECTED FREQUENCY OF ANTIGENIC VARIANTS

Monoclonal antibody	Dilution of ascites used	Detected frequency of variants(log_{10})*	HI ratio§ (wild type / variant)	Preinoculation neutralization titer	
				variant	wild type
XY102	1:10	<-7.1	——**	——	1:230,000
	1:40	-5.1	10 - 13	<1:1,000	
	1:160	ND‡			
XY106	1:10	-5.3	5∿6	<1:100	1:450,000
	1:40	-4.7	4∿7	1:100	
	1:160	ND†			
XY101	1:10	-5.4	>10	<1:10	1:160,000
	1:40	-5.4	>10	<1:10	
	1:160	ND			
XY107	1:10	4.4	>10	<1:100	1:100,000
	1:40	4.1	>10	<1:100	
	1:160	4.0	>10	<1:100	
	1:640	-3.9	>10	<1:100	
	1:2560	ND			
XY108	1:10	-5.1	>13	<1:100	1:100,000
	1:40	-5.1	>13	<1:100	
	1:160	-5.2	>13	<1:100	
	1:640	ND			
XY104	1:10	-5.5	>14	<1:10	1:256,000
	1:40	-5.4	>14	1:10	
		ND			

Legend of Table XV:

*The frequencies of variants for the same monoclonal antibody at different antibody concentrations were detected in the same experiment.

§$\log_2$ of HI ratio measured by RDE treated monoclonal antibody versus X31 virus and versus the variants.

†Not detectable due to the presence of unneutralized wild type virus.

**Not detectable due to the lack of visible plaques in the presence of XY102 monoclonal antibody at ascites fluid dilution 1:10.

routine assay); whereas in the other assay system, monoclonal antibodies were removed after the adsorption period. Thus, in the first method antibody is present both before infection and during subsequent cycles of replication, whereas with the second method, antibody is present only at the time that the first cycle is initiatd. As can be seen in Table XVI, two patterns emerged when these two assays of numbers of variants were employed. For example, the frequencies of detected variants with XY101 were identical when antibody was present during both pre-inoculation and post-inoculation periods and when antibody was present only during the pre-inoculation period. Conversely with XY103, the frequency of detected variants was more than 100-fold higher when antibody was present only during the pre-inoculation period as compared to the frequency detected when antibody was present during both periods. This marked differences in the frequency of variants detected in the presence of XY103 in the two assay systems may be related to data shown in Table XVII. Whereas the pre-inoculation neutralization titers of XY101 and XY103 are equivalent, XY103 has a six fold higher titer in the post-inoculation plaque reduction assay. Thus, it is possible that in the case of XY103, some variants which escaped neutralization during the pre-inoculation period were sufficiently inhibited during subsequent multicycle replication when antibody was continuously present, that they were not detectable as plaque variants. In contrast, in the case of XY101, the post-inoculation plaque reducing activity was not capable of inhibiting replication sufficiently to prevent these variants from producing visible plaques.

From the studies reported in section B, it is evident that the detected frequencies of antigenic variants in cloned virus population can

TABLE XVI

DETECTED FREQUENCIES OF ANTIGENIC VARIANTS IN DIFFERENT ASSAY SYSTEMS USING MONOCLONAL ANTIBODIES XY101 and XY103

Monoclonal antibody	Frequency of detected variants (log_{10}) pre- only*	pre-+post-†
XY101	-4.7	-4.7
XY103	-5.2	-7.3

*Antibody was present during the pre-inoculation period only

†Antibody was present during both pre-inoculation and post-inoculation periods.

TABLE XVII

PRE-INOCULATION NEUTRALIZATION TITERS AND POST-INOCULATION PLAQUE REDUCTION TITERS OF XY101 and XY103

Monoclonal antibodies	Pre-inoculation neutralization titer	Post-inoculation plaque reduction titer
XY101	180,000	90,000
XY103	160,000	550,000

be influenced by antibody avidity, antibody concentration and the assay system employed.

C. STUDIES OF THE BIOLOGICAL ACTIVITY OF MONOCLONAL ANTIBODIES IN MICE

Because monoclonal antibodies XY101 and XY103 behaved differently in assays of post-inoculation plaque inhibiting activity in tissue culture, we thought it would be interesting to determine whether the two monoclonal antibodies have different biological activities in mice challenged with X31 virus. Two experiments were designed for this purpose:

(i) The two monoclonal antibodies were inoculated into separate groups of mice one day before virus infection in concentrations (1:10 dilution of ascites) calculated to produce equivalent serum HI titers. Groups of these mice were challenged with different dilutions of X31 virus by aerosol to determine the dilution of virus at which fifty percent of the mice were infected, as determined after inoculation of ground lung suspensions into embryonated eggs 3 days after challenge. As shown in Table XVIII both groups of passively immunized mice were relatively resistant to the initiation of X31 virus infection as compared to unimmunized controls. However, there was no significant difference in the concentration of virus needed to infect the two groups of passively immunized mice.

(ii) Monoclonal antibodies were inoculated into mice four hours after virus infection (the details of this procedure are described in the Materials and Methods section). In this instance, monoclonal antibodies were present only after virus infection had been initiated, and thus is equivalent to the post-inoculation plaque reduction assay in tissue culture. As can be seen in Table XIX, XY101 and XY103 has similar

TABLE XVIII

MID_{50} OF X31 VIRUS IN MICE PASSIVELY IMMUNIZED WITH DIFFERENT MONOCLONAL ANTIBODIES PRIOR TO CHALLENGE

Monoclonal antibody	MID_{50}*
XY101	$10^{-2.6}$
XY103	$10^{-2.1}$
PBS	$10^{-3.9}$

*Estimated dilution of X31 seed virus required for aerosol infection of 50% of mice. Results obtained after exposure of different groups (8/group) of mice to serial 5-fold dilutions of virus.

TABLE XIX

EFFECTS OF PASSIVE IMMUNIZATION OF MICE WITH MONOCLONAL ANTIBODIES ONE DAY AFTER INFECTION WITH X31 VIRUS

Exp. no.	Monoclonal antibody	Virus titers in lung (EID_{50},log_{10}) day 3	day 7	Lung lesions (%) day 7
I*	XY101	7.0 ± 0.4†	<3.3 ± 1.5	20
	XY103	7.4 ± 0.5	<2.5 ± 1.8	20
	PBS	7.7 ± 0.2	5.9 ± 0.8	50
II**	XY101	4.6 ± 0.7	<2.8 ± 0.1	37.5
	XY103	4.1 ± 0.5	<2.7 ± 0	17.5
	PBS	7.2 ± 0.2	ND§	all dead

* Mice were passively immunized with a 1:10 dilution of ascites fluid

** Mice were passively immunized with a 1:5 dilution of ascites fluid

† Mean + S.D.; calculated from EID_{50} of 5 mice in each group

§ Not determined because all mice in this group were dead on day 7

effects on both pulmonary titers and the extent of lung lesions 7 days after infection. In another experiment similar to the one just described, the passive administration of two fold higher concentrations of XY101 or XY103 four hours after infection was associated with significantly lower virus titers in both groups compared to controls three days after challenge, but there were no significant differences in virus titers in recipients of XY101 as compared to recipients of XY103.

D. CROSS-REACTIVE IDIOTYPY AMONG MONOCLONAL ANTIBODIES TO INFLUENZA VIRUS HEMAGGLUTININS

Cross-reactive idiotype of monoclonal antibodies to PR8 and B/Lee hemagglutinins was studied using anti-idiotype antisera.

(i) Cross-reactive idiotype among monoclonal antibodies to PR8 virus hemagglutinin

The cross-reactive idiotypy among monoclonal antibodies directed against PR8 virus HA obtained both from the same fusion and from different fusions were studied using either homologous or syngeneic anti-Id sera. When homologous anti-P28 antiserum was used to detect shared idiotypes among P8, P20 and P28 (these three monoclonal antibodies were from the same fusion), extensive cross-reactivity was demonstrated (Table XX) in both HI and competition RIA assays, despite the fact that these three monoclonal antibodies had been shown to be directed to different antigenic determinants. In contrast, no cross-reactivity with monoclonal antibodies to B/Lee HA was observed. Similarly, in assays of direct binding of ^{125}I-labeled P8, P28 and B109 (50,000 cpm of 10^6 cpm/μg) to plates coated with anti-P28 antibody (20 μg/ml), the results which were obtained were 1376, 9893 and 220 cpm, respectively.

TABLE XX

REACTIVITY OF MONOCLONAL ANTIBODIES TO PR8 VIRUS HA WITH A/J ANTI-P28 ANTISERUM ASSAYED BY HEMAGGLUTINATION INHIBITION AND RADIOIMMUNOASSAY

Monoclonal antibodies	HI titers* (log_2)	Competition RIA (μg/ml) of purified antibodies giving 50% inhibition)†
P8	6	<.01
P20	>12	<.01
P28	6	<.01
B109	0	>100

*Dilution of purified monoclonal antibodies (original concentration =1 mg/ml) which inhibits the binding of A/J anti-P28 antibody to SRBC-P28.

†Concentration of purified monoclonal antibody which cause 50% reduction of binding of ^{125}I-labeled P28 (50,000 cpm of $\sim 10^6$ cpm/μg) to 20 μg/ml anti-P28 coated wells.

In further study with monoclonal antibodies to PR8 HA, we found that binding of anti-P28 to monoclonal antibodies against PR8 HA was inhibited by purified PR8 viral antigen (Table XXI). These results indicate that anti-P28 recognizes determinants on monoclonal antibodies closely associated with their antigen binding sites.

In other studies, homologous or syngeneic anti-id antiserum against monoclonal antibodies obtained from different fusions was used to detect shared idiotypes, (P8, P20 and P28 are from one fusion; PY102 and PY105 are from another fusion; PY202, PY207, PY210 and PY211 are from a third fusion) in HA assay. It is evident from Table XXII that the monoclonal antibodies from different fusions also share cross-reactive idiotypes.

(ii) Cross-reactive idiotypy among monoclonal antibodies to B/Lee virus hemagglutinin

The presence of individual idiotypes (IdI) and cross-reactive idiotypes (IdX) on B/Lee specific monoclonal antibodies was studied using syngeneic anti-Id antisera (i.e. BALB/c anti-B118, anti-B142 and anti-B147). (B118, B142 and B147 are from the same fusion.)

The IdI was studied in three systems as follows (Table XXIII): (a) Anti-B142Id antibodies versus B142: In this system, we found that only B109 shared the idiotypic determinants of B142 detected in both HI and competition RIA assays. In addition, when direct SRIA (data not shown in the table) were done with ^{125}I-labeled B/Lee specific monoclonal antibodies (50,000 cpm of ~10^6 cpm/μg) and anti-B142Id antibody (3 μg/ml), over 3,000 cpm of ^{125}I-B142 and ^{125}I-B109 were bound to the plates whereas less than 400 cpm of other radiolabeled monoclonal antibodies were bound. (b) Anti-B118Id antibodies versus B118: In this system we found that B142 and B147 share some idiotypic determinants of

TABLE XXI

INHIBITION BY PR8 VIRUS OF BINDING OF MONOCLONAL ANTIBODIES AGAINST PR8 HA TO ANTI-P28Id ANTIBODY

^{125}I-labeled monoclonal antibodies	µg/ml of purified PR8 virus giving 50% inhibition*
P8	.9
P20	.8
P28	.4

*Concentration of purified PR8 virus which causes a 50% reduction of binding of ^{125}I-labeled monoclonal antibodies (50,000 cpm of 10^5 cpm/µg) to 40 µg/ml anti-P28 Id antibody coated wells

TABLE XXII

HA TITERS OF HOMOLOGOUS AND SYNGENEIC ANTI-ID ANTIBODIES IN THE PR8 VIRUS SYSTEM

Anti-Id sera	Sheep erythrocytes coated with monoclonal antibodies: SRBC-PY202	SRBC-PY207	SRBC-PY210	SRBC-PY211
BALB/c anti-P20	3‡	1	0	4
BALB/c anti-P28	3	1	4	6
BALB/c anti-PY105	2	0	0	3
BALB/c anti-PY202	7	0	0	3
A/J anti-P8*	4	4	0	3
A/J anti-P20*	5	5	0	4
A/J anti-PY102*	2	0	2	0
A/J anti-PY105*	2	0	0	1
A/J anti-PY207*	3	6	0	3

*All A/J anti-Id sera were first diluted 1:10 in saline, then adsorbed with one-tenth volume of 3mg/ml MOPC167 (a BALB/c Ig)before use in the HA assay

‡HA titers are expressed in $\log_2$ units

TABLE XXIII

INDIVIDUAL IDIOTYPES (IdI) EXPRESSED ON DIFFERENT MONOCLONAL ANTIBODIES TO B/LEE VIRUS HA DETECTED BY HEMAGGLUTINATION INHIBITION AND RIA ASSAYS

	anti-B142 + B142		anti-B118 + B118	anti-B147 + B147
Inhibitors	HI*	RIA†	HI	HI
B109	8	5	ND	ND
B118	0	>100	8	0
B123	0	>100	ND	ND
B127	0	>100	ND	ND
B141	0	100	ND	ND
B142	7	1	2	0
B147	ND	>100	3	3
P8	0	>100	0	0

ND = Not determined

*Dilution of purified monoclonal antibodies (original concentration = 1 mg/ml) which inhibites the binding of anti-Id antibodies to SRBC coupled with monoclonal antibodies against B/Lee HA.

†Concentration of purified monoclonal antibody which cause 50% reduction of binding of ^{125}I-labeled B142 (50,000 cpm of $\sim 10^6$ cpm/µg) to 3 µg/ml anti-B142 coated wells.

B118 as assessed by HI assays. (c) Anti-B147Id antibodies versus B147: No other monoclonal antibodies studied expressed the IdI of B147.

These results suggest that some monoclonal antibodies (e.g. B147) may bear a true IdI whereas some individual idiotopes (e.g. idiotypes detected by anti-B142Id antiserum) can be shared by only few monoclonal antibodies.

To study the cross-reactive idiotypes (IdX), a hemagglutination inhibition assay that allowed the investigation of shared idiotypes (IdX) and minimized the role of IdI's was used. This technique was used previously in studies of the IdX of a human IgM myeloma with rheumatoid activity in which cross-reactive idiotypes was initially discovered (Kunkel, et al., 1973) and thereafter in the study of IdX of murine myeloma proteins (Lieberman, et al., 1975).

The IdX's of monoclonal antibodies were studied by hemagglutination inhibition in three systems:

(a) Inhibition of binding of ^{125}I-labeled anti-B118Id and anti-B142Id antibodies to SRBC-B109: as shown in Table XXIV, both systems all monoclonal antibodies expressed the IdX (i.e. inhibited binding).

(b) Inhibition of binding of radiolabeled anti-B147Id antibodies to SRBC-B109: In this system only 3 out of 6 monoclonal antibodies expressed the IdX.

These results indicate that some of the monoclonal antibodies bear a true IdI, some express idiotypes shared by few members and some bear a true IdX.

The binding of syngeneic anti-Id antibodies to monoclonal antibodies to B/Lee HA was inhibited by purified B/Lee viral antigen (Table XXV). These results indicate that anti-Id antibody recognizes antigenic

TABLE XXIV

CROSS-REACTIVE IDIOTYPES (IdX) EXPRESSED ON DIFFERENT MONOCLONAL ANTIBODIES TO B/LEE VIRUS HA DETECTED BY HEMAGGLUTINATION INHIBITION ASSAY

	HI titers (log_2)*		
	Anti-Id antiserum + ligand		
Inhibitor	anti-B118 + SRBC-B109	anti-B142 + SRBC-B109	anti-B147 + SRBC-B109
B109	4	5	6
B118	5	3	0
B123	5	3	0
B127	4	3	0
B141	4	4	4
B142	4	5	6
P8	0	0	0

*Dilution of purified monoclonal antibodies (original concentration = 1 mg/ml) which inhibits the binding of various anti-Id antisera to SRBC coated with B109 monoclonal antibody.

TABLE XXV

INHIBITION BY B/LEE VIRUS OF BINDING OF MONOCLONAL ANTIBODIES TO ANTI-IDIOTYPE ANTIBODIES

^{125}I-labeled monoclonal antibodies	µg/ml of purified B/Lee virus giving 50% inhibition*	
	Anti-B118	Anti-B142
B109	ND	.7
B118	.6	.9
B142	.9	1.0

ND = Not determined

*Concentration of purified B/Lee virus which causes a 50% reduction of binding of ^{125}I-labeled monoclonal antibodies (50,000 cpm of $\sim 10^6$ cpm/µg) to 10 µg/ml anti-B118 or anti-B142 antibody coated wells

determinants on monoclonal antibodies closely associated with their antigen binding sites.

It should be noted that in the B/Lee virus system among HA specific monoclonal antibodies obtained from hybridoma from a single fusion, sharing of cross reactive idiotypes was more readily detected than when monoclonal antibodies obtained from a different fusion were employed in the same system. Thus, B109, B118, B123, B141 and B142 are monoclonal antibodies derived from one fusion, while BY103 and BY104 are monoclonal antibodies obtained from another fusion. (PY105 is monoclonal antibody against PR8 virus HA.) In all three systems, (anti-B123+^{125}I-B109; anti-B123+^{125}I-B142; anti-B142+^{125}I-B123), BY103 expressed less cross-reactive idiotypy (IdX) than B109, B118, B123, B141 and B142, and BY104 expressed no detectable IdX (Table XXVI). The possible explanation and significance of this phenomenon will be speculated upon in the Discussion section.

E. IDIOTYPES EXPRESSED DURING THE PRIMARY AND SECONDARY ANTI-VIRAL RESPONSES OF MICE IMMUNIZED WITH B/Lee VIRUS

When primary and secondary immune sera from mice immunized with B/Lee virus were tested for their anti-viral hemagglutinating inhibiting activity, typical primary and secondary responses were observed (Figure 2). During the primary immune response, a lag period of about 5 days was followed by increasing anti-viral antibody titers which reached a peak 10 to 20 days after immunization and then slowly declined. During the secondary immune response, antibody titers increased after a very brief lag phase (less than 3 days) and reached a plateau by day 7 after booster immunization.

The same immune sera were used in four HI assay systems to study idiotypic expression during primary and secondary immune responses. As illustrated in Figure 3, the mean serum titer of cross-reactive idiotype detected by anti-B118 and B123 was 2.3 ± .5 before immunization, reached a peak of 4.2 ± .9 on day 5 during the primary immune response, and dropped gradually thereafter. Following secondary immunization, there was an initial decline in the titers of this idiotype (possibly due to formation of complexes between idiotype and viral antigen). Thereafter, titers rose slowly and reached a peak of 4.7 ± .5 on day 20 after secondary immunization.

The titer of cross-reactive idiotype detected by anti-B118 and B142 was 1.2 ± .7 before immunization. During the primary immune response, it reached a plateau on day 5 of 3.0 ± .6, and began to decline on day 20 after immunization. During the secondary immune response, the same idiotype, following an initial decline rose rapidly in titer, reaching a lower peak than was seen in the primary response and declined slowly thereafter.

The HI titer of cross-reactive idiotype detected by anti-B142 and B109 was 1.8 ± .8 before immunization. After primary immunization, it rose immediately reached a peak of ~4.0 between day 5 and day 10 and then declined. In contrast to the other idiotypes, this idiotype did not increase significantly in titer during the first 30 days of the secondary immune response.

The HI titer of cross-reactive idiotype detected by anti-B147 and B109 was 3.5 ± .5 in non-immunized animals. A slight decrease during the primary immune response was observed. However, the titer of the same

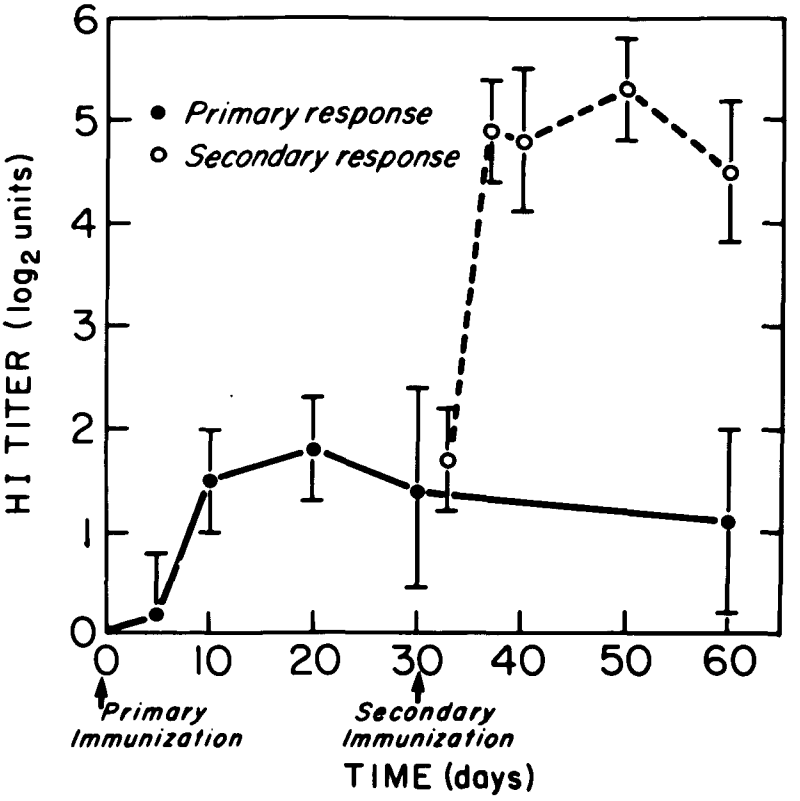

Figure 2. Antiviral hemagglutinating inhibiting titers during primary and secondary responses of mice immunized with B/Lee virus. Titers are expressed as the geometric means of the reciprocal of the serum dilution causing inhibition of 32-64 hemagglutinating units of B/Lee virus, and are calculated from individual titrations of sera of at least six mice.

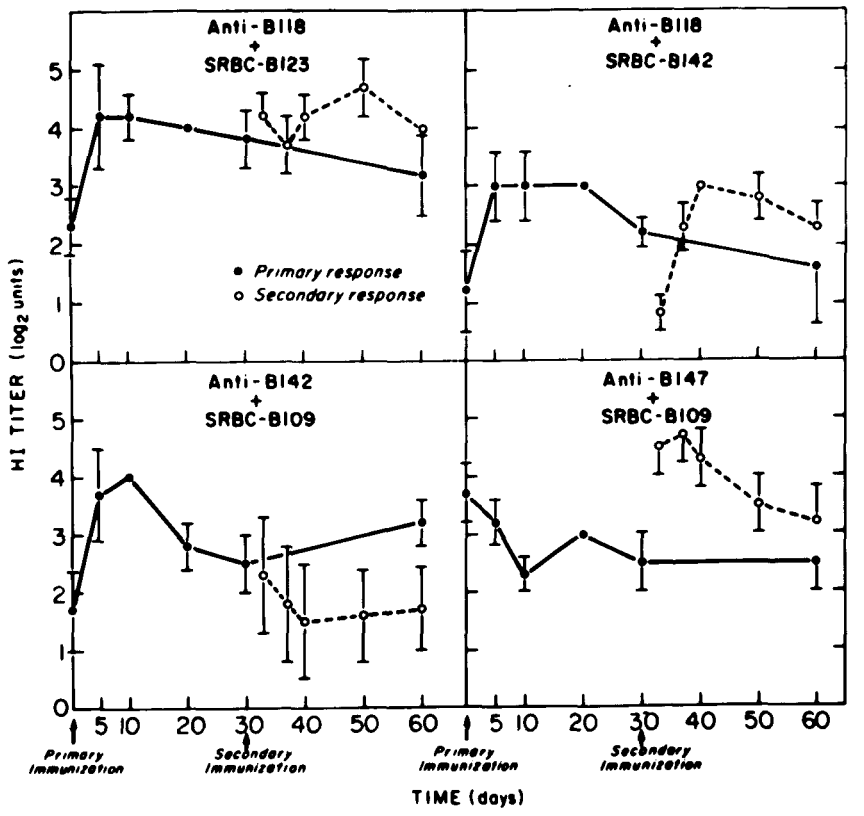

Figure 3. IdX expressed during primary and secondary responses of mice immunized with B/Lee virus. Sera were as used in Figure 2. Two-fold dilutions of sera were used to inhibit the binding of anti-Id antisera to SRBC coated with monoclonal antibodies.

idiotype increased promptly after secondary immunization and then decreased gradually.

To determine whether or not the IdX expressed early in the secondary response was attributable to antibody molecules with anti-HA specificity, we pooled the sera obtained 3 and 7 days after secondary stimulation and passed the pool through a B/Lee-sepharose affinity column to separate the fractions which bound and did not bind to B/Lee virus. The two fractions then were tested in a conventional anti-viral HI assay and for IdX expression in HI assay using the anti-B147 and SRBC-B109 system. All of the anti-viral activity was recovered in the fraction which bound to virus, but this fraction contained only 11% of the HI activity in the anti-B147 SRBC-B109 system. Conversely, the unbound fraction which lacked detectable anti-viral HI activity was responsible for 89% of the IdX expression. These results suggest that the majority of the IdX detected in the secondary response was due either to a parallel set of Ab_1 or to Ab_3. In an effort to distinguish between these two possibilities, we searched for the presence of Ab_2 (which could have provided the stimulus for Ab_3 synthesis) in sera obtained 20 and 30 days after primary immunization. Using direct hemagglutination assay with SRBC coated with B109, we were unable to detect anti-Id (i.e. Ab_2) antibodies in the late period of the primary immune response when IdX titers were declining.

F. ONTOGENY OF IMMUNE RESPONSE TO B/Lee VIRUS

BALB/c mice at birth and 7, 14, 21 and 28 days of age were immunized with purified B/Lee virus (10 μg protein/mouse), and their serum anti-viral responses as well as cross-reactive idiotype responses were determined 5 days after immunization. As can be seen in Table XXVII,

TABLE XXVI

CROSS-REACTIVE IDIOTYPES (IdX) EXPRESSED ON MONOCLONAL ANTIBODIES SPECIFIC FOR B/LEE VIRUS HA DETECTED BY COMPETITION RIA

Monoclonal antibody	Concentration(μg/ml) required for 50% inhibition of binding of ^{125}I-Id to anti-Id*		
	anti-B123 + ^{125}I-B109	anti-B123 + ^{125}I-B142	anti-B142 + ^{125}I-B123
B109	1	30	0.01
B118	0.7	30	0.4
B123	0.08	0.27	1.0
B141	9	ND	6.6
B142	7	0.23	0.3
BY103	100	70	30
BY104	100	100	100
PY105	100	ND	ND

*Affinity purified BALB/c anti-Id(30μg/ml) was used to coat microtiter plates. ^{125}I-monoclonal antibodies (50,000 cpm in 25 ul) were added followed by the addition of different concentrations of affinity purified cold monoclonal antibodies.

ND = Not detected

TABLE XXVII

ONTOGENY OF ANTI-VIRAL HI RESPONSES IN BALB/c MICE

Age of mice (days)	Number of mice studied	Anti-viral HI titer* (log_2)
newborn	5	0
7	6	2.7 ± 0.5
14	5	4.0 ± 0.7
21	5	3.2 ± 0.8
28	5	4.2 ± 0.9
adult pre-immune sera	4	0

*Two-fold dilutions of RDE-treated sera were used to inhibit the hemagglutination of type "O" human erythrocytes by 16∿32 hemagglutinating units of B/Lee virus. The titer is presented as the reciprocal (log_2) of the highest dilution of RDE-treated sera that causes inhibition of hemagglutination ± S.D.

anti-viral HI antibody to B/Lee virus was not detectable in newborn mice immunized with B/Lee virus, but were found in approximately the same titers in all other age groups studied.

The same sera was used to measure expression of IdX using affinity purified syngeneic anti-B123 and radiolabeled B142 by competition RIA. Undiluted sera and 1:3, 1:10, 1:30 and 1:100 dilutions of individual sera (except for newborn mice) from sensitized mice of different ages was used as inhibitors. Inhibition of binding was not detected at all with diluted sera (data not shown), but as seen in Table XXVIII, mice 7 days of age or older expressed very low levels of this cross-reactive idiotype following immunization. Similarly, when the same sera was employed in HI assay using SRBC coated with B142 and A/J anti-B123, extremely low levels of cross-reactive idiotype was detected in mice of one week of age or older (Table XXVIII).

These results suggested two things: First, the idiotype we investigated is at least a very minor component of the B/Lee virus response; second, in the development of responsiveness to B/Lee virus, this particular idiotype is expressed very early and matures at approximately the same time as the anti-B/Lee HA response.

G. GENETIC CONTROL OF ANTI-HEMAGGLUTININ RESPONSE AND CROSS-REACTIVE IDIOTYPE RESPONSE TO B/Lee VIRUS

(i) Anti-hemagglutinin response

Various strains of mice were immunized with B/Lee virus (10 μg protein/mouse), and serum anti-viral HI titers during primary and secondary responses were determined. None of the normal mouse sera had detectable amount of anti-viral HI activity. All mice studied including

TABLE XXVIII

ONTOGENY OF CROSS-REACTIVE IDIOTYPE RESPONSES IN BALB/c MICE

Age of mice (days)	Number of mice studied	Cross-reactive idiotype response detected by Competition RIA*	HI of A/J αB123+SRBC-B142‡
newborn	5	8.7§	1
7	6	19.3 ± 10.2	1
14	5	18.3 ± 6.5	2
21	5	21.6 ± 5.2	2
28	5	27.3 ± 5.2	2
adult preimmune sera	4	7.2 ± 7.7	1

*Data represent % inhibition ± S.D. BALB/c anti-B123 antibody (30μg/ml) were used to coat the wells. ^{125}I-radiolabeled B142 (50,000 cpm) were added to each well.

‡Pooled sera were employed to detect HI titers of A/J αB123 and SRBC-B142.

§Result of pooled sera from 5 neonatal mice

H-2^d (i.e. BALB/c, C.B20, BAB.14, and DBA/2J), H-2^b (i.e. BALB.B and C57BL/6), H-2^k (i.e. AKR/J, CE/J, C3H/J and CBA/J), H-2^q (i.e. DBA/1J and SWR/J), H-2^a (i.e. A/J), H-2j (i.e. RI1IS/J) and H-2^u (i.e. PL/J) haplotypes had detectable anti-viral HI antibody at all time intervals studied after both primary and secondary immunization, and the responses to secondary immunization were generally higher than those following primary immunization (Table XXIX). Furthermore, the two congenic strains, BALB/c (H-2^d) and BALB.B (H-2^b) made equivalent anti-viral HI responses. Thus, we conclude that all strains of mice studied are capable of responding following immunization with B/Lee virus, and that the expression of anti-B/Lee antibody is not controlled by MHC genes.

(ii) Cross-reactive idiotypic response

The same sera from these 15 strains of mice were employed to investigate the genetic control of the cross-reactive idiotype response to B/Lee virus immunization using the same heterologous interaction of anti-B123Id and ^{125}I-labeled B142 as was used in the ontogeny study. Again, low levels of inhibition were obtained only with the undiluted immune sera and no inhibition was observed in 3-fold diluted sera (data not shown). Again, these results indicated that this particular IdX system represents a minor component of the anti-B/Lee response. Individual normal mouse sera from all strains were included as controls. As can be seen in Table XXX, most of the pre-immune sera did not cause significant level of inhibition of binding of ^{125}I-B142 to anti-B123Id. However, in spite of the large variation among individual sera, at different time periods and among strains, the immune sera from all strains including various MHC haplotypes and Igh-C allotypes contained detectable levels of this cross-reactive idiotype after primary

TABLE XXIX

ANTI-VIRAL RESPONSE OF DIFFERENT STRAINS OF MICE FOLLOWING IMMUNIZATION WITH B/LEE VIRUS

Strain	MHC type	Igh-C type	Number of mice studied	Anti-viral HI titer (log_2) of sera*			
				Primary response		Secondary Response	
				day 0	day 10	day 20	day 7	day 20
BALB/c	d	a	4	0	3.0±0	2.2±0.4	5.6±0.5	5.0± 0
C.B20	d	b	3	0	2.3±1.2	2.5±0.7	4.7±1.5	4.0±1.0
BAB.14	d	V_H a C_H b	7	0	3.2±0.4	2.7±0.5	4.3±1.9	4.5±1.0
DBA/2J	d	c	3	0	3.6±0.9	3.2±0.8	5.0±1.0	5.3±0.7
BALB.B	b	a	3	0	3.0±1.2	2.3±1.3	4.8±0.4	3.8±0.7
C57BL/6	b	b	5	0	ND	4.2±0.4	5.0±1.0	5.0± 0
AKR/J	k	d	4	0	3.5±0.6	3.2±0.4	4.8±0.5	4.3±0.5
CE/J	k	f	3	0	4.8±0.5	5.5±0.6	5.3±0.5	6.0±0.8
C3H/J	k	j	5	0	4.4±0.5	5.0±0.7	5.0± 0	4.2±0.6
CBA/J	k	j	5	0	4.3±0.5	4.8±0.4	5.8±0.8	5.6±0.9
DBA/1J	q	c	3	0	3.0±0	3.4±0.5	5.4±0.9	4.6±0.7
SWR/J	q	c	4	0	5.7±0.6	4.3±0.5	5.0±0	5.0±0
A/J	a	e	5	0	4.5±0.6	4.4±0.9	6.4±0.5	6.2±0.4
RIIIS/J	r	g	4	0	4.5±0.6	4.4±0.9	6.4±0.5	5.5±0.4
PL/J	u	j	4	0	3.3±1.0	2.7±0.6	4.3±1.0	4.7±0.5

*Mean of HI titers ± S.D.

TABLE XXX

CROSS-REACTIVE IDIOTYPE RESPONSE OF DIFFERENT STRAINS OF MICE DETECTED BY COMPETITION RIA

Strain	MHC	Igh-C		Percent inhibition of binding of ^{125}I-B142* primary response day 0	primary response day 10	primary response day 20	secondary response day 7	secondary response day 20
		V_H	C_H					
BALB/c	d		a	7.2 ± 7.7	19.3 ± 7.5	12.1 ± 5.0	12.3 ± 3.3	16.1 ± 9.0
BAB.14	d	a	b	14.0 ± 10.0	28.3 ± 5.1	20.6 ± 5.1	26.8 ± 6.2	16.3 ± 11.2
DBA/2J	d		c	4.0 ± 0.9	2.5 ± 0	21.0 ± 8.8	23.9 ± 9.7	14.2 ± 6.2
BALB.B	b		a	6.4 ± 8.5	12.5 ± 12.2	14.5 ± 3.3	19.2 ± 8.2	27.7 ± 3.6
C57BL/6	b		b	11.0 ± 4.0	ND‡	14.4 ± 5.2	8.7 ± 5.6	20.3 ± 6.3
AKR/J	k		d	-1.2 ± 11.8	24.2 ± 9.3	18.2 ± 7.7	29.0 ± 5.1	24.4 ± 6.7
CBA/J	k		j	-1.9 ± 8.5	-0.1 ± 6.1	30.1 ± 7.7	12.2 ± 2.5	22.1 ± 3.8
DBA/1J	q		c	7.8 ± 11.5	19.0 ± 3.8	8.7 ± 0.3	17.8 ± 0.5	23.4 ± 1.5
SWR/J	q		c	7.9 ± 8.0	2.7 ± 9.3	22.3 ± 3.9	16.9 ± 8.5	ND‡
A/J	a		e	7.8 ± 3.0	27.8 ± 2.8	10.5 ± 6.7	15.4 ± 8.2	24.1 ± 9.1
RIIIS/J	r		g	11.9 ± 2.8	15.6 ± 4.9	16.3 ± 5.9	20.4 ± 12.6	24.4 ± 5.6
PL/J	u		j	-6.9 ± 5.8	13.6 ± 14.7	17.6 ± 7.8	16.6 ± 2.2	24.2 ± 5.0

Legend of Table XXX:

*Percent inhibition ± SD. Affinity chromatography purified BALB/c anti-B123 (30µg/ml) was used to coat the wells. After FCS was applied to each well to block empty sites, 25 µl of undiluted sera and 25 µl of 50,000 cpm ^{125}I-B142 were added to each well.

‡Not determined.

immunization and/or after secondary immunization. To further confirm that the expression of IdX in mice immunzied with B/Lee virus is not affected by MHC or Igh-C genes, IdX in pooled sera of several strains of mice were studied in HI assays. As can be seen in Table XXXI all strains of mice studied expressed the cross-reactive idiotypes detected in the A/J anti-B123+SRBC-B142 system and BALB/c anti-B142+SRBC-B118 system after primary or secondary immunization or both.

From the above studies, we conclude that expression of these IdXs in mice immunized with B/Lee virus are not regulated by MHC genes or linked to Igh-C genes.

H. THE EXPRESSION OF B/Lee IDIOTYPE IN MICE IMMUNIZED WITH NATURAL VARIANTS OF B/Lee VIRUS

One of the unique features of influenza viruses is that surface proteins, hemagglutinin and neuraminidase, undergo antigenic variation frequently in nature. Epidemic and pandemic influenza results from antigenic drift and antigenic shift, respectively, and from the subsequent ineffectiveness of pre-existing antibodies to older strains. Among influenza B viruses, only antigenic drift has been demonstrated (see the Introduction section). In other words, the hemagglutinins of all B type viruses are serologically cross-reactive to varying degrees. In this part of the study, we were interested in determining whether BALB/c mice immunized with natural variants of influenza B virus would express the IdX of anti-B/Lee HA monoclonal antibodies. The viruses selected in this study covered a broad-time spectrum of isolates (from 1954 to 1972). Sera obtained after priming and secondary immunization of mice with different variants (10 μg viral protein) were tested with respect to anti-viral HI antibody titers against the immunizing virus and

TABLE XXXI

CROSS-REACTIVE IDIOTYPE RESPONSE OF DIFFERENT STRAINS OF MICE DETECTED BY HI ASSAYS

	Primary response						Secondary response			
	day 0		day 10		day 20		day 7		day 20	
Strain	A*	B†	A	B	A	B	A	B	A	B
BALB/c	1	0	ND§	ND	2	3	2	4	2	4
BAB.14	0	0	0	0	2	0	2	0	2	0
BALB.B	ND	ND	0	3	2	2	3	3	3	3
AKR/J	0	0	ND	ND	4	1	3	4	ND	ND
CBA/J	0	0	0	4	2	3	ND	ND	ND	ND
SWR/J	0	0	ND	ND	ND	ND	2	3	0	0
A/J	ND	ND	0	3	3	3	2	3	2	3
RIIIS/J	ND	ND	ND	ND	3	4	2	4	ND	ND
PL/J	ND	ND	0	0	4	4	ND	ND	ND	ND

*$\log_2$ of serum dilution which inhibited agglutination of SRBC-B142 by A/J anti-B123Id.

†$\log_2$ of serum dilution which inhibited agglutination of SRBC-B118 by BALB/canti-B142.

§not determined because of insufficient serum

against B/Lee virus. As can be seen in Table XXXII, during the primary response, only sera from mice immunzied and B/Md/59 virus showed cross-reactivity with B/Lee/40 in HI assay. However, during the secondary response sera from mice immunized with all four variants showed detectable cross-reactivity with B/Lee/HA, and the extent of cross-reactivity could be arranged according to the following order: B/Md/59>B/HK/8/72>B/Vict/70≥B/GL/54.

The same sera were then examined by competition RIA for the presence of the cross-reactive idiotype fo B/Lee monoclonal antibodies in the anti-B123 and ^{125}I-labeled B142 system. As shown in Table XXXIII, no cross-reactive idiotype could be detected in preimmune sera, but the cross-reactive idiotype was detected at low levels in both the primary and secondary sera of all mice. It is worth mentioning that among sera obtained 10 days after primary immunization, sera from mice immunized with B/Md/59 gave the highest anti-B/Lee HI titers (Table XXXII and the same sera seemed to contain relatively more of the cross-reactive idiotype detected by anti-B123Id and ^{125}I-B142. Furthermore, the relative amount of this cross-reactive idiotype in day 7 sera of the secondary response could be arranged in the following order: B/Md/59>B/HK/8/72>B/Vict/70 and B/GL/54. This ranking is similar to the titers of HI antibody against B/Lee virus in secondary sera.

I. THE DETECTION OF CROSS-REACTIVE IDIOTYPE IN VARIOUS ANIMAL SPECIES

The expression of IdX of B/Lee monoclonal antibodies in primary and secondary immune sera of various animal species, including chicken, rat, rabbit and guinea pig, immunized with B/Lee virus was studied by competition RIA. In addition, the expression of the same IdX in human

TABLE XXXII

ANTIVIRAL HEMAGGLUTINATING INHIBITING ANTIBODY TITERS IN MICE IMMUNIZED WITH DIFFERENT NATURAL VARIANTS OF INFLUENZA B VIRUS

Virus used in immunization	HI titer(log_2) against immunizing virus			HI titer(log_2) against B/Lee/40 virus		
	day 0	primary day 10	secondary day 7	day 0	primary day 10	secondary day 7
B/GL54	0	3.4 ± 1.7*	5.4 ± 2.1	0	0	0.8 ± 1.1
B/Md/59	0	3.4 ± 0.2	5.8 ± 1.1	0	1.0 ± 0	2.8 ± 0.8
B/Vict/70	0	2.0 ± 0.7	3.4 ± 0.3	0	0.2 ± 0.4	1.2 ± 0.7
B/HK/8/72	0	3.0 ± 0.7	6.2 ± 0.8	0	0	1.8 ± 0.4

*Value represents mean ± S.D. for 5 mice

TABLE XXX III

EXPRESSION OF CROSS-REACTIVE IDIOTYPE IN SERA OF MICE IMMUNIZED WITH DIFFERENT NATURAL VARIANTS OF INFLUENZA B VIRUS

Virus used in immunization	Idiotype response measured by inhibition of RIA*		
	day 0	primary day 10	secondary day 7
B/GL/54	2.3 ± 5.0	14.2 ± 4.7	21.4 ± 6.1
B/Md/59	9.7 ± 12.5	26.4 ± 7.2	40.1 ± 8.9
B/Vict/70	-3.8 ± 10.0	23.1 ± 10.2	20.6 ± 6.9
B/HK/8/72	-7.0 ± 9.5	15.1 ± 7.8	30.7 ± 6.4

*Values represent percent inhibition ± S.D. for 5 mice in each group BALB/c anti-B123 (30μg/ml) was used to coat the wells. Radiolabeled B142 (25λ of 50,000 cpm) was added to each well along with 25λ unidluted sera.

cord blood serum and human adult sera was also studied. Unexpectedly, extensive non-specific inhibition was observed with pre-immune sera of all of these animals (50% to 70% inhibition) when undiluted sera were used. When these sera were diluted to avoid this non-specific inhibition, specific inhibition by immune sera was also not detectable. Therefore, we attempted to purify these sera over a B/Lee affinity column and then used the purified sera in competition RIA in the anti-B123 and ^{125}I-B142 system. The results (data not shown) of this latter study indicated that there was no detectable IdX of B/Lee monoclonal antibodies in any of the sera of the animals investigated.

IX. DISCUSSION

A. FACTORS WHICH MAY INFLUENCE THE DETECTED FREQUENCY OF ANTIGENIC VARIANTS.

Different methods, such as nucleic acid hybridization (Scholtissek et al., 1978; Palese et al., 1981), oligonucleotide sequencing (Porter et al., 1979; Winter et al., 1981) peptide mapping (Laver and Webster, 1971), and peptide sequencing (Air et al., 1981) have been used to investigate genetic changes in influenza viruses. But, since many genetic changes in the HA and NA genes do not result in detectable antigenic changes, serological methods must be included in the analysis in order to detect antigenic variants. In this respect, monoclonal antibodies have a distinct advantage over heterologous immune sera in that they can be applied to detect specific virus variants restricted to a particular antigenic determinant. Monoclonal antibodies have been used in several laboratories to determine the frequencies of antigenic variants in cloned virus populations (Laver et al., 1979; Yewdell et al., 1989; Lubeck et al., 1980; Portner et al., 1980) and frequencies in the range of $10^{-4.1}$ to $<10^{-8.8}$, have been reported from different laboratories. One of the main purposes of the present studies was to investigate the possible factors that might influence the detected frequency of antigenic variants.

Monoclonal antibodies against PR8, X31 and BLee virus hemagglutinin were grouped to be either against the same (or overlapping) antigenic determinants or against different antigenic domains according to two criteria:

i. If the frequencies of antigenic variants detected in the presence of pairs of monoclonal antibodies were equivalent to that

observed in the presence of only one monoclonal antibody, these two monoclonal antibodies were defined to be against the same or functionally overlapping determinants. If the frequencies detected in the presence of two monoclonal antibodies were the product of those detected in the presence of each of one monoclonal antibodies separately, the two monoclonal antibodies must be against distinct antigenic areas. This criterium can distinguish monoclonal antibodies which recognize distinct antigenic domains, but the weakness of this criterium is that it tends to group topographically related antigenic determinants into overlapping determinants because of steric hindrance.

ii. If variants selected in the presence of one monoclonal antibody no longer reacted or reacted at a significantly changed titer in HI assay with the other monoclonal antibodies, these two monoclonal antibodies are defined to be directed against the same or functionally overlapping determinants. This criteria will distinguish two very closely located antigenic determinants. The weakness of this criterium is that monoclonal antibodies with very different avidities against the same (or overlapping) determinants may be grouped into antibodies which are specific for different determinants. Occasionally, mutations at one epitope may affect the binding of monoclonal antibody specific for another epitope through conformational changes.

In the PR8 virus system, the three monoclonal antibodies P8, P20 and P28 are clearly against distinct determinants according to these two criteria. In the BLee virus system, monoclonal antibodies B109, B118, B127, B141 and B142 are against the same or overlapping determinants by both criteria: monoclonal antibodiy B147 also seems to be against determinants which overlap with the former determinant(s). In the X31

virus sytem, the grouping of monoclonal antibodies is difficult to establish because of asymmetry of reactivity and differences in reactivity with different variants selected by a single monoclonal antibody. These two phenomenon were also observed by other investigators (Gerhard, et al., 1980; Gerhard, et al., 1981). Thus, the definitive mapping of antigenic determinants recognized by monoclonal antibodies has to be done by sequencing of variants.

To simplify the study, we selected five monoclonal antibodies against the same (or overlapping) determinants on B/Lee HA to compare the frequencies of antigenic variants detected when each was used in selection and found an inverse relationship between antibody avidity and frequency of detected variants (Table IX). This relationship was further confirmed when the detected frequencies of variants with a single monoclonal antibody (B118) and different laboratory and natural variants was examined (Table X). This latter observation is particularly noteworthy because in this study the same monoclonal antibody was employed to determine the frequencies of variants in different virus populations to which the antibody bound with different avidities. Thus, any possible effects of differences in the epitope recognized or of antibody concentration or isotype can be excluded.

Furthermore, it is worth mentioning that only B118 (the highest avidity antibody) cross-reacted with B/Md/59 and B/HK/73 viruses despite the fact that the other 4 monoclonal antibodies B109, B127, B141 and B142 recognize the same or an overlapping antigenic determinant on the HA of B/Lee virus. Parenthetically, this last observation supports the suggestion by Russel et al., (1979) that an antibody population to a particular determinant may be specific or cross-reactive depending upon

the avidity of the antibody populations present. However, these data do not exclude the possibility that some naturally occurring variants contain antigenic determinants which are identical to those in previous strains and hence might properly be called "common" determinants as opposed to "strain specific" determinants (reviewed by Kilbourne et al., 1971).

A similar relationship between antibody avidity and detected frequencies of antigenic variants was observed when monoclonal antibody X146 was tested with X31 virus and with laboratory selected variant viruses XY106m4, XY105m5 and XY108m5. As shown in Table XIV, again there was an inverse relationship between the avidity with which a monoclonal antibody binds to a particular virus and the detected frequencies of antigenic variants. These observations are compatible with the speculation that in the presence of high avidity antibody a high proportion of variants present in cloned virus seeds do not escape neutralization and hence are not detected as variants, whereas in the presence of low avidity antibody these same mutant viruses are not neutralized and can be detected as variants. The absence of a recognizable inverse correlation between avidity and frequency of detected variants in the PR8 virus system might be related to the fact that the monoclonal antibodies employed in this study are directed against distinct antigenic sites (Table III). In the absence of definitive data, it is only possible to speculate as to why the detected frequencies of antigenic variants with monoclonal antibodies directed to different epitopes may be quite different. Among the hypothetical possibilities are: (a). Differences in mutational frequencies at different sites; (b). Mutational events outside of antigenic domains may

cause conformational changes which afffect some epitopes more than others; (c). Because of differences in the location of different antigenic domains on the HA, substitutions in different epitopes may have markedly different effects on the neutralizing activity of monoclonal antibodies specific for each antigenic site.

In the X31 virus system, some of the factors that influence differences in the frequencies of antigenic variants detected by eleven monoclonal antibodies are not obvious (Table XIII). For example, XY101, XY104, XY106, XY105, X188, XY102, and XY107 are all IgG antibodies; they have similar binding constants to virus measured by solid phase RIA, and their concentrations in ascites fluids are not significantly different. However, the frequencies of antigenic variants detected in the presence of XY101. XY104, XY106 and X188 are in the range of $10^{-5.2}$-$10^{-5.8}$; that of XY102 is less than $10^{-7.1}$ (at least about 100 fold lower than average); whereas, those of XY105 and XY107 are $10^{-4.4}$ and $10^{-4.6}$ respectively. However, as seen in Table XIII, these differences in detected frequencies of antigenic variants detected by these seven monoclonal antibodies are not attributable to the class of monoclonal antibody, monoclonal antibody concentration in ascites fluid or to antibody avidity. Similarily, X202, has the lowest avidity but the frequency of antigenic variants detected in the presence of X202 is not lower than those observed with most of the other monoclonal antibodies. We speculate, that these differences in detected frequencies of antigenic variants are most probably related to differences in the particular epitopes involved in virus HA and monoclonal antibody interaction although some of the monoclonal antibodies mentioned above may be against partially overlapping antigenic areas functionally.

On the other hand our studies did demonstrate that in some instances at least, antibody concentration could be a factor influencing the detected frequencies of antigenic variants. For example, the extremely low frequency of antigenic variants detected in the presence of undiluted RDE treated XY102 increased 100 fold when the ascites was diluted 1:4 (Table XV). This result suggests that in some instances variants may not be detected if antibody is present in great excess (i.e., if present in high concentrations) the antibody may still be capable of neutralizing variants which otherwise would escape. In most other instances, a decrease in antibody concentration either did not significantly influence the detected frequency of variants or resulted in insufficient concentration of antibody to completely neutralize wild type virus. The reason(s) why changes in antibody concentration had different effects on detected frequency of variants for different monoclonal antibodies is (are) not clear.

Another factor which appeared to play a role in assays of detected frequencies of variants was related to differences which were obtained when different assay systems were employed. Thus, it was interesting to note that the frequency of variants detected when XY101 was present only during the preinoculation period and that observed when it was present both during preinoculation and postinoculation periods are the same (Table XVI). On the other hand, the detected frequency of variants observed when XY103 was present both during preinoculation and postinoculation periods was much lower than that observed when it was present only during the preinoculation period. These results can be correlated with neutralizing and plaque reduction properties of XY101 and XY103 shown in (Table XVII). It can be seen that XY101 has a relatively

low postinoculation plaque reduction titer. Therefore, it is likely that XY101 is unable to react with variants which have escaped neutralization during the preinoculation period; on the other hand, XY103 has a relatively high post-inoculation plaque reduction titer and thus may be capable of inhibiting multicycle replication of variants which escaped neutralization during the preinoculation period. (It should be noted that these differences in relative titers of XY101 and XY103 in preinoculation neutralization and postinoculation plaque reduction assay were observed in repeated experiments).

We cannot definitively explain why two monoclonal antibodies with similar preinoculation neutralization titers have different postinoculation plaque titers. However, it should be noted that XY101 and XY103 are not directed to the identical antigenic determinant of the X31 virus HA and it is possible that antibody populations specific for different determinants may vary with respect to their relative activities in the two assay systems. It is also possible that the relatively higher concentration of XY103 in ascitic fluid may be a factor. In any case, it seems clear that in the case of XY103 the assay system employed had a significant effect on the frequency of detected variants.

To summarize, the factors that may influence the detected frequency of antigenic variants are antibody avidity, antibody concentration, the assay system employed and probably the specific sites to which monoclonal antibodies bind.

B. IDIOTYPY OF CLONAL RESPONSES TO INFLUENZA VIRUS HEMAGGLUTININ

Although the structural basis for antigenic variation of influenza A virus HA molecules has been elucidated by recent sequence analysis of variants (Laver et al., 1979; Webster and Laver, 1980; Winter et al.,

1982; Gething et al., 1980; Verhoeyen et al., 1980; Fang et al., 1981; Sleigh et al., 1981; Wiley et al., 1981), as yet, little is known about the clonal nature of the anti-HA responses which confer immunity to infection. The availability of monoclonal antibodies to influenza virus HA's and of technique for raising anti-idiotype antibodies provide a potentially powerful tool to investigate the idiotypes expressed during the immune response and for determining whether specific antibodies to antigenic variants are derived from a diverse set of clonotypes or from the progeny of common ancestor clones.

Analysis of the idiotypes of the three PR8 HA specific monoclonal antibodies P8, P20 and P28 (these monoclonal antibodies against different determinants on P28 HA) with homologous anti-Id antisera revealed extensive cross-reactivity as assessed in HI and RIA assays (Table XX). This unusual idiotypic cross-reactivity has been found previously in other systems among antibodies to different antigen molecules (Oudin and Cazenave, 1971) and among antibodies of unknown specificities (Oudin and Cazenave, 1981; Bona et al., 1979). The finding of shared IdX among P8, P20 and P28 monoclonal antibodies despite their different antigenic specificities and the fact that they are of different IgG subclasses suggests that the IdX is encoded by (a) germline gene(s).

With respect to the mechanism responsible for shared IdX among monoclonal antibodies of different IgG subclasses specific for different HA determinants, several explanations can be entertained:

(i) The clones associated with different antibody specificities but similar idiotype specificities are derived from one ancestral clone or V gene ancestor. During embryonic development and clonal expansion, after antigen stimulation, somatic mutations may occur in V-region genes

so that different antibody specificities are generated while idiotype specificities remain relatively unchanged (Bona and Hiernaus, 1981).

(ii) The V_H region of immunoglobulin is encoded by separated V and J genes. During B cell maturation, V and J regions are juxtaposed so it is possible that antibodies bearing different antigen binding specificities may have identical J segments (Bona and Hiernaus, 1981; Schilling et al., 1980).

(iii) During B cell maturation, different minigenes (D genes) which code for 1-7 amino acids are inserted between V and J genes. This D gene may contribute to the distinct antigen binding specificities, but antibodies may still have cross-reactive idiotypic determinants (Ju et al., 1980; Kabat, 1980).

(iv) The IdX represent(s) regulatory idiotype(s) which are capable of becoming dominant idiotypes because it is these determinants that stimulate Id specific regulatory cells (Bona et al., 1980). The data obtained in the present experiments do not permit us to distinguish among these possibilities.

The study of the idiotypes expressed by monoclonal antibodies specific for B/Lee HA (Tables XXIII and XXIV) including B109, B118, B123, B127, B141, B142 and B147, demonstrated that these monoclonal antibodies bear three categories of idiotypic determinants:

(i) idiotypes borne by a single monoclonal antibody, equivalent to an individual antigenic specificity of of a myeloma protein (Kunkel et al., 1963), i.e., an individual idiotype.

(ii) idiotypic determinants shared by a few but not all of the monoclonal antibodies.

(iii) cross-reactive idiotypes born by monoclonal antibodies specific for the same or overlapping antigenic determinants on B/Lee HA.

It should be emphasized that the B/Lee specific monoclonal antibodies employed are of different subclasses (Table VII). Moreover, although they are directed to overlapping antigenic determinants, the avidity with which individual monoclonal antibodies bind to B/Lee has been found to vary over a wide range. Hence, the IdX observed among these antibodies is not attributable to their being products of a particular clonotype. Nevertheless, the finding of IdX among antibody populations with overlapping but nonidentical specificities is not unexpected and could be explained by any of the 4 mechanism discussed above.

It should be noted that in our studies we observed that monoclonal antibodies resulting from different fusions but directed against the same virus HA had less cross-reactive idiotypy than monoclonal antibodies obtained from the same fusion. This observations may be explained by one (or more) of the following mechanisms:

(a) During clonal expansion after antigen stimulation, different dominant idiotypes (i.e., regulatory idiotypes) are expressed at different stages of maturation. Each fusion happened to catch clonotypes characteristic of a particular maturation stage.

(b) During clonal expansion after antigen stimulation, different somatic mutations or genetic rearrangements may occur in V region genes so that the V domains of monoclonal antibodies from different fusions are less alike than those from the same fusion.

In our studies, we also noted that both with monoclonal antibodies to PR8 and B/Lee viruses binding to anti-idiotype antibody was inhibited

by prior incubation of monoclonal antibodies with virus (Tables XX and XXV). These results are compatible with the assumption that the idiotypic determinants are closely associated with the combining site. However, this does not mean that idiotypic determinants which no longer react with anti-idiotypes after interaction with virus are necessarily combining site associated. Indeed, Kunkel et al., (1976), showed that binding of anti-Rh antibodies to a large antigen such as red blood cells can lead to the disappearance of some idiotypic determinants and the appearance of other antigenic determinants that are different from those associated with the combining site.

Both persistence and variation of idiotypes during the immune response have been demonstrated in other systems. Thus, it was shown in humans, that idiotypes of anti-Rh antibodies can persist for several years and in mice that the idiotype of anti-galactan antibodies can persist for 150 days (Saint Martin et al., 1978; Bona, 1981). In contrast, it was shown in rabbits that some idiotypes of anti-S. typhi (Oudin and Michel, 1969), and -M. lysodeiktieus (Brown and Rodkey, 1979) or anti-pheynlarsonate (McDonald and Nisonoff, 1976) detected early after immunization disappear and are replaced by new idiotypes in the late phases of the immune response. In mice, in the hen lysozyme system, the IdX-HEL was found only in the secondary immune response (Metzger, et al., 1980).

In general, the results obtained in our studies of the idiotypes expressed during immune responses of mice immunized with B/Lee virus are in accord with these observations in that both persistence and variation of different idiotypes during primary and secondary immune responses were observed.

First, it should be noted that the IdI detected by anti-B147 and SRBC-B147 was not detectable in the sera of mice after primary or secondary immunization with B/Lee virus. Some cross-reactive idiotypes were detectable in the sera of mice prior to immunization. The presence of IdX in non-immunized mice could be explained either by antibodies with unknown specificities which could share the IdX of monoclonal antibodies, or alternatively by the existence in the nonimmunized mmice of a small amount of antibody to B/Lee HA which is undetectable by antiviral HI assay but is detectable by PFC assay (Reiss and Schulman, 1980).

The IdX's detected by anti-B118 and SRBC-123, and by anti-B118 and SRBC-B142 were expressed during both primary and secondary immune responses.

In contrast, the IdX detected by inhibition of binding of anti-B142 to SRBC-B109 was observed only during the primary response. This absence of idiotype during the secondary response could be explained by

(i) the induction of an anti-id antibody during the secondary response which clears the corresponding idiotype from the blood, or

(ii) the appearance of suppressor T cells specific for the idiotype which suppress the expression of this idiotype.

The level of idiotype detected by anti-B147 and SRBC-B109 was very high in non-immunized mice, but was reduced after primary immunization. On the other hand, this idiotype was expressed immediately after secondary stimulation. The high titers of this idiotype during the secondary response could be explained by one (or more) of the following mechanisms.

(i) expression during the secondary response but not the primary response of an Ab_1 (id) having antigen specificity and bearing the IdX;

(ii) expression of a parallel Ab_1 which bears the IdX but lacks antigen specificity;

(iii) induction of Ab_3 (anti-anti-id) which bears the IdX and either does or does not have antigen binding specificity. Since the majority of IdX expressed early in the secondary response was found in the serum fraction which did not bind to virus, Ab_1 and Ab_3 with antigen specificity may be excluded as the major source of IdX bearing antibody seen in the secondary response. Although we could not detect anti-itiodype (Ab_2) antibody in sera obtained late in the primary response, we cannot exclude the possibility that Ab_2 antibody in concentrations too low to be detected with the assay system employed, in the form of complexes, or sequestered in lymphoid organs was present in quantities sufficient to induce an Ab_3 response. Hence, we are unable at present to distinguish whether the preponderance of IdX observed in the secondary response using the anti-B147 SRBC-B109 system is due to a parallel Ab_1 set or to Ab_3.

The ontogeny of IdX expression in sera of BALB/c mice immunized with B/Lee virus was investigated by competitive RIA in the anti-B123Id and ^{125}I-B142 system. Only low levels of inhibition were observed even when undiluted serum samples were used as inhibitors, suggesting that the idiotype we studied represent minor components of the clonal repertoire to B/Lee virus. However, the data support the hypothesis that the maturation of expression of IdX studied follows that of the anti-B/Lee response.

Cancro et al., (1979) have shown that in young mice there are only few clones specific for influenza viral HA, and that most of these clones are lost in adult mice. Our data show that the cross-reactive idiotype

we studied is present early (before 12 days of age) and persists in adult life. This further support the idea that this particular idiotype is produced by clonotypes which appear at low frequency.

Studies on the ontogeny of idiotype expression in earlier studies revealed that different idiotypes are activated at different times during life.

X24TdX of anti-galactan response (Bona, 1981) and 384Id or anti-LPS response (Bona, 1981) are activated at birth or soon after; 460Id of the anti-TNP response, T151d of the anti-PC response (Signal et al., 1976) and IdX of the anti-Ars response (Nutt et al., 1979) are activated early during post-natal life (approximately 5 days to less than 2 weeks of age); whereas the IdX or the anti-β (2->1) fructosan response (Bona et al., 1979) and the anti-α (1->6) dextran response (Howard and Hale, 1976; Fernandez and Moller, 1978) are activated later in mice (approximately at 4 weeks of age). The results together with those presented here support the concept that V genes of immunoglobulin are sequentially activated during postnatal life.

Our studies also demonstrated that the IdX which was studied can be detected during immune response to B/Lee virus among all strains of mice regardless of H2 or Igh-C haplotype indicating that this IdX is of germ line origin.

Such interstrain IdX which is not linked to allotypes nor influenced by major histocompatibility genes has also been observed in other systems. Mushinshi and Potter (1977) showed that β6- GALBMPIdX was detected in different strains of mice during different stages after immunization with gum ghatti. Ju et al., (1979) showed that GA-1 idiotype was detected in 21 inbred strains of mice immunized with GAT.

Our results also provided evidence that the same minor IdX was expressed in mice immunized with natural variants of influenza B virus. These results are also compatible with the concept that this IdX is of germ line origin and that somatic mutations or combinational events may result in B cell clones producing antibody of different specificities which retain shared idiotypic determinants.

To summarize, in the studies of idiotypy among monoclonal antibodies to influenza virus hemagglutinin we have found that: (a) cross-reactive idiotypy among monoclonal antibodies to influenza virus hemagglutinin is not restricted to monoclonal antibodies directed to the same or overlapping epitopes, monoclonal antibodies to distinct antigenic determinants on the hemagglutinin of PR8 virus also show cross-reactive idiotypy. In addition, this sharing of idiotypes is not restricted to monoclonal antibodies obtained from a single fusion, although monoclonal antibodies obtained from different fusions tend to share less cross-reactive idiotypy. These observations lead us to speculate that the cross-reactive idiotypes detected may reflect regulatory idiotypes mediated by idiotype specific T_H cells, but other possibilities are equally plausible. (b) Some of these cross-reactive idiotypes are present in BALB/c mice after primary immunization with influenza virus, some are present after the secondary immunization or both. (c) The cross-reactive idiotype is present at low levels in all strains of mice studied indicating that it is probably of germ line origin. (d) Detection of the IdX in the response of BALB/c mice to natural variants of B/Lee virus suggests that the same germ line genes are employed in the response to variants.

X. SIGNIFICANCE

1. Detected frequencies of variants can be influenced by antibody avidity, antibody concentration, the method of assay and by other as yet undefined factors.

2. Cross-reactive idiotype among monoclonal antibodies to different sites and in heterogeneous sera obtained after immunization with variants suggests that idiotype regulation may be an important factor influencing immune responses to influenza viruses and that clones producing antibody to different sites and to altered epitopes are derived from common precursor clones.

XI. APPENDIX: ABBREVIATIONS

A-CHO	group A streptococcal carbohydrate
ADCC	antibody dependent cellular cytotoxicity
A/J	an inbred mouse strain
AKR/J	an inbred mouse strain
Ars	p-azophenylarsonate
ATS	anti-thymocyte serum
BAB.14	an inbred mouse strain
BAB.B	a congenic mice of BALB/c
BALB/c	an inbred mouse strain
BL	bacterial levar
B/Lee virus	influenza B/Lee/40 virus
C.B20	an inbred mouse strain
CBA/J	an inbred mouse strain
C57BL/6	an inbred mouse strain
CD-1	an inbred mouse strain
CE/J	an inbred mouse strain
C3H/J	an inbred mouse strain
CGAT	a common idiotype of anti-GAT antibodies
CMI	cell mediated immunity
cpm	counts per minute
D1.LP	a congenic mouse strain of DBA/1J
DBA/1J	an inbred mouse strain
DBA/2J	an inbred mouse strain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary strand of DNA made of RNA template

DNP	2,4-dinitrophenyl
DTH	delayed type hypersensitivity
EID_{50}	50% egg infectious doses
ELISA	enzyme-linked immunoabsorbent assay
FCS	fetal calf serum
GAT	a synthetic polypeptide of ($L\text{-}Glu^{60}$, $L\text{-}Ala^{30}$, $L\text{-}Tyr^{10}$)
GT	a synthetic polypeptide of ($L\text{-}Glu^{50}$, $L\text{-}Tyr^{50}$)
H chain	heavy chain of immunoglobulin
H-2	major histocompatibility complex of mouse
HA	hemagglutinin glycoprotein
HA titer	hemagglutination titer
HAT	a selective medium containing hypoxanthine, aminopterin and thymidine
HbA1	A chain of human hemoglobin
HbS	human sickle cell hemoglobin
HEL	hen's egg lysozyme
HI titer	hemagglutination inhibition titer
^{125}I	a radioactive iodine
Id	idiotype
IdI	individual idiotype
IdX	cross-reactive idiotype
Ig	immunoglobulin
Igh-C	allotype of immunoglobulin heavy chain
J segment	a DNA segment located between V and C segements of immunoglobulin gene
K	binding constant
KLH	keyhole limpet hemocyanin

L chain	light chain of immunoglobulin
LPS	lipopolysaccharide
Ly-1, Ly-2	cell surface antigens of T cells
M protein	matrix or membrane protein
MDCK cells	Madin-Darby canine kidney cells
MHC	major histocompatibility complex
MID_{50}	50% mouse infectious doses
NA	neuraminidase
ND	not done
NI test	neuraminidase inhibition test
NP	nucleoprotein
NS	non-structural protein
PA	acidic polymerase (P) protein of influenza virus
PB1	the larger of the two basic P proteins of influenza virus
PB2	the smaller of the two basic P proteins of influenza virus
PBS	phosphate buffered saline
PC	phosphocholine
PEG	polyethylene glycol
PL/J	an inbred mouse strain
PR8 virus	influenza A/PR/8/34 virus (HINI)
P3X63Ag8	a myeloma cell line of BALB/c origin
RBC	red blood cells
RDE	receptor destroying enzyme
RIA	radioimmunoassay
RNA	ribonucleic acid
RNP	ribonucleoprotein
RIIIS/J	an inbred mouse strain

S.D.	standard deviation
SP2/0	a myeloma cell line of BALB/c origin
SRBC	sheep red blood cells
SWR/J	an inbred mouse strain
TNP	2,4,6-trinitrophenyl
X31 virus	a recombinant influenza virus of A/Aichi/2/68 (H3N2) x A/PR/8/34 (HINI)

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