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DIVERSITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL INFLUENZA VIRUS VARIANTS AND ATTEMPT TO ELICIT AN ANTI-INFLUENZA IMMUNE RESPONSE BY IMMUNIZATION WITH MONOCLONAL ANTI-IDIOTOPE ANTIBODIES AND ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT

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by

Michael A. Reale

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1985

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Aug 2, 1985
Date

Jerome L. Schulman, M.D.
Chairman of Examining Committee

August 23, 1985
Date

Terry A. Krulwich, Ph.D.
Executive Officer

Constantin A. Bona, M.D., Ph.D.

Peter Palese, Ph.D.

Patricia Mongini, Ph.D.

Nolan H. Sigal, M.D.

Supervisory Committee

The City University of New York

I. ABSTRACT

DIVERSITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL INFLUENZA VIRUS VARIANTS

AND

ATTEMPT TO ELICIT AN ANTI-INFLUENZA IMMUNE RESPONSE BY IMMUNIZATION WITH MONOCLONAL ANTI-IDIOTOPE ANTIBODIES

AND

ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT

by

Michael A. Reale

Advisor: Jerome L. Schulman, M.D.

DIVERSITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

A panel of monoclonal antibodies specific for a corresponding panel of sequentially selected variants of influenza A/PR/8/34 virus has been established. Though the monoclonal antibodies are paratypically distinct, idiotypic relatedness has been observed. Two cross reactive idiotypes have been defined that are associated with the 7183 and S107 V_H gene families, respectively. The V_K21 group of light chains is highly represented among these antibodies. Studies of the idiotypy, and V_H and V_L genes of this panel of monoclonal antibodies suggests that both germ line encoded diversity and somatic diversifying mechanisms have contributed to

their paratypic differences.

ATTEMPT TO ELICIT AN ANTI-INFLUENZA IMMUNE RESPONSE BY IMMUNIZATION WITH MONOCLONAL ANTI-IDIOTOPE ANTIBODIES

Monoclonal anti-idiotope antibodies directed to influenza specific monoclonal antibodies have been characterized in terms of their specificity. While 2 of these anti-idiotope antibodies(SN3-1A and 63-4) define idiotopes that are largely confined to PR8 virus immune sera, SN3-9A is more cross reactive in that it defines idiotopes used in both PR8 and X-31 virus immune sera. SP3-5A is an extremely cross reactive anti-idiotope reagent. Sera from mice immunized with PR8, X-31, A/Singapore(H2N2), A/Houston(H1N1), A/Chicken(H5N1) and B/Lee virus all express the SP3-5A defined idiotope. Furthermore, the SN3-9A and SP3-5A defined idiotopes were shown by adsorption studies to reside on hemagglutinin and/or neuraminidase specific antibodies.

ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT

The humoral response to PR8 virus was examined in the CBA/N and C_3J.xid strains of mice, both of which bear an X-linked genetic defect(xid), and in strains lacking this defect. Hemagglutination inhibiting antibody titers and measurement of virus specific antibodies by radioimmunoassay indicated that the xid defect does not impair the production of an adequate anti-influenza

antibody response. However, investigation of the isotypes of PR8 virus specific antibodies disclosed a relative decrease in the levels of IgG3 and IgG1 in the *xid*-bearing strains. This was observed after both intraperitoneal immunization and aerosol infection. The isotype differences were not reflected in the susceptibility of these strains to influenza virus infection.

II. ACKNOWLEDGEMENTS

I would like to thank Lynn, without whom this would not have been possible.

I would like to thank my family for their constant encouragement and love.

I would like to extend my sincerest appreciation to Dr. Jerome Schulman for being a patient and encouraging teacher and friend. He has provided a solid foundation and example for my future development as a scientist.

I would like to thank Dr. Constantin Bona for his guidance and a constantly stimulating interaction.

I would like to thank Dr. Peter Palese, who taught me a great deal in the little time we spent together.

I would like to extend my sincerest thanks to Mrs. Mary Ann Diliberti and Mrs. Sue Lin for their technical assistance and their friendship.

I would like to extend a special thanks to Ms. Kaye Leitzinger for her enormous technical help and also for a friendship that brightened my day.

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V. AIMS

A. Previous findings in this laboratory(Liu et al., 1981, 1983; Moran et al., 1984) have shown extensive cross reactive idiotypy among influenza specific monoclonal antibodies from different animals that are directed to distinct epitopes. Therefore, the hypothesis was formulated that monoclonal antibodies specific for sequential PR8 virus variants might be even more likely to share an idiotype, indicating a somewhat restricted genetic origin and consequently the importance of somatic events in generating their paratypic diversity.

A panel of monoclonal antibodies specific for a corresponding panel of sequentially selected PR8 virus variants was established. Based on a characterization of their idiotypic relationships and also their expressed V_H and V_L genes, we have attempted to account for the paratypic diversity of these monoclonal antibodies.

B. One goal of this laboratory is to elicit an anti-influenza immune response by immunization with anti-idiotype antibodies. A panel of monoclonal anti-idiotope antibodies specific for influenza specific monoclonal antibodies was produced. We characterized these monoclonal anti-idiotope reagents and also attempted to use them as immunogens.

C. The *xid* defect is an X-linked defect found in CBA/N mice that interferes with the development of a B cell subset which displays the Lyb5 surface antigen(Scher, 1982), resulting in unresponsiveness to particular T-independent antigens. We examined the humoral immune response to influenza A/PR/8/34 virus in mouse strains with and without this defect in order to determine if this developmental defect in any way alters the anti-influenza response.

VI. INTRODUCTION

A. INFLUENZA VIRUSES

1. STRUCTURE - GENERAL FEATURES

Influenza viruses are small(80-120 nm diameter), RNA containing, enveloped animal viruses belonging to the family orthomyxoviridae. Their lipid bilayer membrane is acquired from the host cell during budding and from it radiate spikes of glycoproteins -- the viral hemagglutinin and neuraminidase. The viral core consists of the M_1 protein, which underlies the lipid bilayer, and a ribonucleoprotein complex, consisting of the segmented, single-stranded RNA genome and the associated nucleoprotein and 3 polymerase proteins (Reviewed by Lamb, 1983).

2. VIRAL PROTEINS - STRUCTURE AND FUNCTION

The hemagglutinin comprises more than 90% of the spikes(Drzeniek et al., 1968). It is involved in attachment to the host cell surface(Lazarowitz and Choppin, 1975) and probably also in fusion to lysosomal membranes during the initial stage of virus infection(White et al., 1982).

Neuraminidase comprises about 10% of the spikes. It is involved in the release of progeny virus particles from infected cells(Seto and Rott, 1966) and also the prevention of aggregation of newly formed viral particles(Palese et al., 1974).

Underlying the host-derived lipid bilayer membrane is the matrix(M_1) protein layer. It is the most abundant protein in the virion(33% of the total protein) and is derived from an unspliced transcript of RNA segment 7(Compans et al., 1970)

The ribonucleoprotein(RNP) complex is composed of segmented RNA strands complementary to message, nucleoprotein and 3 P proteins, 2 of which are basic(PB1 and PB2) and 1 of which is acidic(PA). In terms of the function of P proteins, Ulmanen et al.(1981) has suggested that the PB2 protein recognizes the 5'-terminal cap structure(m^7GpppNm) on host mRNAs, and that the PB1 protein initiates transcription via the incorporation of a guanosine residue onto the 3' end of a capped RNA fragment. It has also been speculated that PA is responsible for elongation of the transcript.

Three non-structural proteins of unknown function are encoded in the viral genome. M_2 is derived from a spliced transcript of RNA segment 7 that utilizes a different reading frame than the M_1 protein for translation of its carboxy terminal 97 amino acids(Lamb and Choppin, 1981; Palese et al., 1981). NS_1 and NS_2 are derived from RNA segment 8. NS_2 is derived from a spliced transcript that utilizes a different reading frame than NS_1 for translation of its carboxy terminal 132 amino acids(Lamb et al., 1978). The lengths 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.

3. VIRUS TRANSCRIPTION AND REPLICATION

The segmented RNA genome of influenza virus is of negative polarity. That is, the viral messenger RNA(mRNA) is complementary to the genome or virion RNA(vRNA). The virus infected cell must produce viral mRNA for ultimate translation into the viral proteins, and it must also produce a distinct complementary RNA that can be used to replicate vRNA.

Among non-oncogenic RNA viruses, only influenza virus requires the functioning of the host nuclear RNA polymerase II, the enzyme which synthesizes the precursors to cellular mRNAs(Barry et al., 1962; Lamb and Choppin, 1977), in order to produce viral mRNA. This was shown by the inhibition of virus replication by alpha-amanitin , a specific inhibitor of RNA polymerase II, and also by the demonstration that in mutant cells containing an alpha-amanitin resistant RNA polymerase II, virus replication was also resistant to this drug(Rott and Scholtissek, 1970; Lamb and Choppin, 1977).

An explanation for the RNA polymerase II requirement was provided by 2 findings. Firstly, studies of the transcription reaction catalyzed in vitro by the virion associated transcriptase showed that the production of

Table I. Influenza virus genome RNA segments and their corresponding polypeptides[a]

Segment/ encoded polypeptide	Length (nucs)	Nascent polypeptide length(aa)	Approx. No. molecules/ virion	Remarks
1/PB2	2341	759	30-60	Host cell RNA cap binding; component of RNA transcriptase
2/PB1	2341	757	30-60	Initiation of transcription; possibly endonuclease activity; component of RNA transcriptase
3/PA	2233	716	30-60	Elongation of mRNA chains?; component of RNA transcriptase
4/HA	1778	566	500	Surface glycoprotein(trimer); major antigenic determinant
5/NP	1565	498	1000	Associated with RNA segments to form ribonucleoprotein; structural component of RNA transcriptase
6/NA	1413	454	100	Surface glycoprotein(tetramer); neuraminidase activity
7/M1	1027	252	3000	Major protein component of virus; underlies lipid bilayer
7/M2	1027	96		Spliced mRNA(non-structural protein); function unknown

Table I(cont.).

Segment/ encoded polypeptide	Length (nucs)	Nascent polypeptide length(aa)	Approx. No. molecules/ virion	Remarks
8/NS1	890	230		Non-structural protein; function function unknown
8/NS2	890	121		Non-structural protein; spliced RNA; function unknown

[a]Modified from Lamb(1983)

polyadenylated mRNA did not occur without the addition of a primer, specifically the dinucleoside monophosphates ApG and GpG in the initial studies(McGeoch and Kitron, 1975; Plotch and Krug, 1977). Secondly, it was found that the virion associated transcriptase complex lacks enzymes capable of capping the 5' terminus of the viral mRNA made *in vitro*(Plotch et al., 1978), eventhough it was known that the viral mRNAs isolated from infected cells do contain 5'-terminal methylated cap structures(Krug et al., 1976). Based on these results it was proposed that viral mRNA synthesis *in vivo* also requires a primer, but that the primer is not a dinucleotide but rather an RNA synthesized by RNA polymerase II, and that the 5' cap is derived from this primer RNA.

Indeed, it has been demonstrated that the synthesis of influenza viral mRNA involves a unique interaction with the host cell transcriptional machinery in the nucleus of the infected cell. A viral endonuclease cleaves 5'-terminal fragments from newly synthesized capped(m^7GpppNm containing) cellular RNAs in the nucleus. These are most likely heterogeneous nuclear RNAs(hnRNAs), the precursors of cellular mRNAs(Herz et al., 1981; Krug, 1981; Plotch et al., 1981). These fragments of capped host nuclear RNAs serve as primers to initiate viral mRNA synthesis.

Because the viral mRNAs contain host derived sequences at their 5' ends and lack sequences complementary to the last 17 to 22 nucleotides at the 5' ends of the vRNA segments(Hay et al., 1977; Hat et al., 1980; Krug, 1981), the viral mRNAs are not suitable templates for the synthesis of vRNA. The presumed templates for vRNA replication are full length transcripts of the vRNA segments. These full length transcripts, which lack 3'-terminal polyadenylate(poly A) sequences, comprise only 5% of the viral transcripts synthesized in the infected cell(Hay et al., 1977). The synthesis of the full length transcripts requires the synthesis of one or more virus coded proteins. The initiation of the full length transcripts apparently occurs without a primer(Hay et al., 1982). Also, the termination of transcription that occurs during viral mRNA synthesis 17 to 22 nucleotides from the 5' end of vRNA must be prevented. Synthesis of vRNA that is directed by the full length transcripts is not well understood at the present time.

4. TYPES OF INFLUENZA VIRUSES

Major distinctions among influenza viruses permit their classification into 3 types -- A, B, and C. The M and NP proteins are serologically type-specific (Pereira, 1969). The lengths of the 8 RNA segments of type A influenza viruses are different from the corresponding 8

segments of the B viruses(Desselberger and Palese, 1978) and the 7 segments of the C viruses(Petri et al., 1979). The influenza C viruses lack a neuraminidase gene(Kendal, 1975; Racaniello and Palese, 1979). Hybridization analyses(cDNA-RNA) and more recently direct sequence comparisons(Nakada et al., 1984a,b) have also indicated that extensive sequence homology observed within a type is not seen between types(Palese et al., 1981).

In addition to the gross structural characteristics of influenza viruses, there are more subtle molecular similarities between types. Nucleotide sequencing of the 5' and 3' ends of the genomic RNAs of types A, B and C has revealed a high degree of conservation of the terminal 10 to 20 nucleotides, suggesting a common evolutionary origin(Desselberger et al., 1980). Also, protein sequences of the type A and B hemagglutinins show conserved stretches of amino acids in areas of functional importance. For example, cleavage of the hemagglutinin (HA) precursor into HA-1 and HA-2 subunits results in HA-2 molecules with common amino terminal sequences. This conserved sequence is thought to be important for viral infectivity(Klenk et al., 1975; Lazarowitz and Choppin, 1975; Waterfield et al., 1979).

5. ANTIGENIC VARIATION OF INFLUENZA VIRUSES

A unique characteristic of influenza virus among animal viruses is its ability to rapidly change its

antigenic structure and consequently circumvent the immune response. This is very different from the situation observed with other animal viruses such as polio and measles that remain essentially unchanged antigenically. Though herpes viruses or rhinoviruses may be present as many co-existing serologically variant viruses, populations of variants do not change in the rapid fashion of influenza. Also, influenza virus is more likely to exist as one or a few predominant variant viruses that are then replaced by a new variant(s), rather than co-existence of several variant forms. Only the African trypanosome, a protozoan, also displays such a powerful ability for antigenic variation(reviewed by Borst and Cross, 1982).

Dramatic changes in the antigenicity of the influenza A virus surface glycoproteins -- hemagglutinin and neuraminidase -- are called antigenic shifts(Webster and Laver, 1975). These result in the creation of new surface glycoprotein subtypes and pandemic influenza. Though Influenza B and C viruses do undergo antigenic variation(Palese et al., 1981), it has never been a dramatic, sudden change that could be called an antigenic shift. Certainly, the amount of antigenic variation seen in the influenza B hemagglutinin has never necessitated the designation of distinct subtypes(Chakraverty, 1972). Influenza B viruses are

more often associated with disease in man than the C viruses, though never to the extent of pandemic influenza(reviewed by Air and Compans, 1983). Consequently, this discussion will be concerned primarily with the influenza A viruses.

The major antigenic shifts in the 20th century have been well documented. From 1934 until 1957 influenza A viruses belonging to the H1N1(swine) subtype circulated throughout the world. The H2N2(Asian) subtype was prevalent from 1957 to 1968 when it was replaced by the H3N2(Hong Kong) strains that are currently circulating. In 1977 strains of the H1N1 subtype reappeared, and since then strains of distinct subtypes -- H1N1 and H3N2 -- have been co-circulating for the first time.

There are two general explanations for the phenomenon of antigenic shift that have significant experimental backing. Firstly, reassortment theory states that viruses resident in non-human populations provide genes for a unique hemagglutinin and neuraminidase that in the context of other genes from human strains create new pandemic viruses(Kilbourne, 1968; Hinshaw et al., 1980). In particular, the Hong Kong H3N2 strain has been shown to be a reassortant virus(Laver and Webster, 1973; Scholtissek et al., 1978; Desselberger et al., 1978) in which the hemagglutinin may be of animal origin since it is antigenically similar to

the hemagglutinin of A/Duck/Ukraine/63(H3N8) virus(Fang et al., 1981; Ward and Dopheide, 1981). Secondly, a "new" subtype may arise as the result of reemergence of a previously circulating strain. The H1N1 strain that reappeared in 1977 appears identical in all genes to the virus which caused an influenza epidemic in 1950(Nakajima et al., 1978; Scholtissek et al., 1978). The status of this strain during the 27 year period is obscure.

In interpandemic periods antigenic variation or drift occurs within a subtype. One mechanism responsible for this antigenic drift is the development of point mutations. This process is thought to involve stepwise, cumulative mutations which result in gradual changes in the antigenic structure of viral surface proteins. Although the majority of these mutations are due to base substitutions, it appears that variations may also be due to short deletions or insertions of triplets in the coding region(Krystal et al., 1980; Caton et al., 1982). Subsequent selection in the presence of antibody to the previous strain favors the growth of antigenic variants(reviewed by Webster et al., 1983). Exchange of genes for nonsurface proteins among co-circulating strains has been shown to contribute to genetic variation within a subtype. For example, the H1N1 strains A/Cal/10/78 and A/Aberdeen/v1340/78 derive 4 and 5 genes, respectively, from co-circulating H3N2 strains(Young and

Palese,1979). Whether this exchange of genes for nonsurface proteins effects antigenic variation is unclear, though it has been speculated that it may allow a particular virus to change more rapidly, generating more successful variants.

6. ANTIGENIC DRIFT IN THE INFLUENZA A VIRUS HEMAGGLUTININ

The hemagglutinin is the major antigen of the virus against which neutralizing antibodies are directed(Laver and Kilbourne, 1966). Three technologies -- X-ray crystallography, monoclonal antibodies and rapid gene sequencing -- have converged to provide a precise molecular understanding of its antigenic structure.

The hemagglutinin monomer is derived from the fourth largest RNA segment and is synthesized as a single polypeptide chain which undergoes at least 3 post-translational cleavages. After removal of the amino terminal signal peptide, the molecule is further cleaved, with the removal of one or more intervening residues, to produce 2 polypeptide subunits-- HA-1(MW~50,000) and HA-2(MW~27,000)(Lazarowitz et al., 1971, 1973a,b). These 2 subunits are held together by a disulphide bond(Laver, 1971).

The 3-dimensional structure of the Hong Kong(H3) hemagglutinin has been determined by X-ray crystallographic studies of the bromelain released glycoprotein(Wilson et al., 1981). Because important

structural features such as disulfide bonds are conserved among hemagglutinins of different subtypes(Krystal et al., 1982), hemagglutinins other than H3 probably have similar structures. The hemagglutinin glycoprotein is a trimer composed of 2 structurally distinct regions -- a triple-stranded coiled-coil of alpha-helices that extends 76 angstroms from the membrane and a globular region of antiparallel beta sheet that contains the receptor binding site. While the proximal fibrous stem-like structure anchors the hemagglutinin into the viral lipid envelope and is made up by the HA-2 and part of the HA-1 subunits, the distal globular domain is made up exclusively by the major portion of the HA-1 polypeptide. Based on sequence information on natural and laboratory selected variants from several laboratories, Wiley et al.(1981) identified 4 antigenic sites -- designated A, B, C and D -- on the Hong Kong hemagglutinin. All are located on the distal globular domain.

Because the H1 hemagglutinin of A/PR/8/34 virus is most relevant to studies presented here, the characterization of its antigenic structure will be discussed in more detail. Gerhard et al.(1981) established a panel of 58 anti-hemagglutinin hybridoma antibodies. He then selected variants of PR8 virus by growing the virus in the presence of an excess of a particular monoclonal antibody. Having established a

panel of variant viruses, the reactivity patterns of binding of the monoclonal antibodies to the variant viruses was determined in an RIA. This allowed for the operational definition of 4 distinct antigenic sites on the hemagglutinin. These viruses were later sequenced and it was found, with few exceptions, that the variant viruses differed from the parental PR8 virus by only a single amino acid. When these amino acid changes were considered in terms of the 3-dimensional structure of the hemagglutinin, they clustered into 4 distinct epitopes(Caton et al., 1982), similar to the earlier work with the H3 hemagglutinin.

7. SELECTION OF VIRUS VARIANTS WITH MONOCLONAL ANTIBODIES

Influenza virus variants are selected _in vitro_ by growth of the virus in Madin-Darby canine kidney cells in the presence of an excess of a virus specific monoclonal antibody. Virus plaques that grow out are expanded in eggs and then confirmed that they are variants by their inability to react with the selecting monoclonal antibody in an HI assay or neutralization assay.

Variant viruses generally differ from the parental virus by a single amino acid change(Caton et al., 1982; Webster et al., 1983). They are useful in distinguishing the paratypes of monoclonal antibodies, and they have also been extremely important, in conjunction with X-ray crystallographic studies and sequence analysis, in

rigorously defining antigenic regions on the hemagglutinin(Wiley et al., 1981; Caton et al., 1982).

The frequency of detected variants has been shown to vary over a wide range($10^{-4.4}$ - $<10^{-8.8}$)(Laver et al., 1979; Yewdell et al., 1979; Lubeck et al., 1980; Portner et al., 1980). Though many factors appear to be involved in determining the frequency of variants, it has been shown that antibody avidity, antibody concentration and the method of assay(i.e. pre- or post-inoculation neutralization assay) are important(Liu, 1983).

B. PROTECTIVE IMMUNITY TO INFLUENZA VIRUS INFECTION

There is abundant evidence that specific antibody to the hemagglutinin present at the time of virus challenge is protective, that immune subjects are less likely to become infected, and if infected, they 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-hemagglutinin 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).

Both the humoral response(Virelizer et al., 1974) and the cytotoxic T cell response(Reiss and Burakoff,

1981) to influenza virus are helper T cell(T_H) dependent. The T_H cell repertoire is just beginning to be defined. Hurwitz et al.(1985) determined the specificity of 11 influenza specific T cell hybridomas, presumedly of the helper cell phenotype, on the basis of their proliferative responses in the presence of purified viral proteins -- 3 were directed to the hemagglutinin, 1 to neuraminidase, 4 to the matrix protein(M_1) and 3 to nucleoprotein.

The specificity of T_H cells specific for the hemagglutinin has been further characterized. Anders et al.(1981) demonstrated that hemagglutinin specific T_H populations were cross reactive for hemagglutinins of different subtypes, a cross reactivity not seen serologically. Analyses of responses of human T_H cell lines or clones to synthetic peptides of the H3 hemagglutinin revealed an immunodominant peptide at the carboxy terminus of HA-1, a region distinct from the proposed antibody binding sites(Lamb et al., 1982). Hurwitz et al.(1984) operationally defined 3 epitopes on the hemagglutinin by examing the reactivity patterns of their hemagglutinin specific T cell hybridomas with a panel of PR8(H1N1) variant viruses. These 3 epitopes were distinct from any of the antibody binding regions. The total number of different reactivity patterns of the hemagglutinin specific T cell hybridomas was also

considerably less than has been seen with hemagglutinin specific monoclonal antibodies, possibly indicating that the T_H cell repertoire is smaller than the B cell repertoire.

Cell mediated immune mechanisms also contribute to both immunopathological events and clearance of virus from the respiratory tract in infected animals. Nude(athymic) BALB/c mice have an increased survival time relative to T cell competent BALB/c mice when a dose of influenza virus lethal to both strains is administered(Sullivan et al., 1976). Death after infection with high concentrations of virulent virus was delayed in cyclophosphamide treated(Hurd and Heath, 1975) or anti-lymphocyte serum treated mice(Suzuki et al., 1974). These 3 observations suggest that morbidity and mortality from influenza virus infection may be mediated immunopathologically, specifically an effect of the T cell component of the response. Alternatively, there is also a great deal of evidence that T cells are very important for clearance of virus from the respiratory tract and recovery from infection. For instance, nude BALB/c mice given a dose of virus that is not lethal to T cell competent BALB/c mice continued to die for as long as 2 to 3 weeks after infection and the virus was isolated from both lungs and spleens at these times(Sullivan et al., 1976). When cyclophosphamide

treated mice were infected with an avirulent influenza virus strain, the usually harmless infection was converted into a fatal pneumonic disease(Hurd and Heath, 1975).

Specifically, cytotoxic T cells have been shown to mediate virus clearance and recovery from infection. In adoptive transfer experiments the effector population involved has been shown to be H-2K or H-2D restricted and to bear the Lyt2 surface antigen characteristic of cytoxic T cell populations(Yap et al., 1978). Similar results have been obtained in adoptive transfer experiments employing T cell clones which have cytotoxic activity against infected cells _in vitro_(Lukacher et al., 1984).

With respect to the specificities of cytotoxic T cell recognition, there is evidence that at least 1 component of the response is hemagglutinin specific. This has been definitively shown by Braciale et al.(1984) who observed that some cytotoxic T cell clones were capable of lysing targets constructed by transfection with an SV40-HA vector in which the only influenza virus protein expressed was the hemagglutinin. Some hemagglutinin specific cytotoxic T cell clones are strain specific and do not lyse targets infected with variant viruses, whereas other hemagglutinin specific cytotoxic clones are cross reactive among hemagglutinins of

different subtypes(Braciale, 1977; Effros et al., 1977; Zweerink et al., 1977; Lu and Askonas, 1980). In adoptive transfer experiments the former were shown to increase only strain specific resistance, whereas the latter provided cross reactive protection(Lukacher et al., 1984).

However, there is appreciable evidence that antigens other than hemagglutinin are involved in recognition by cytoxic T cells. Indeed, most cytotoxic T cell clones identified in limiting dilution analyses are specific for to internal virus proteins(Kees and Kramer, 1984). In particular, viral nucleoprotein appears to be the target for a significant proportion of cytotoxic T cell clones. Moreover, some of these clones appear to recognize nucleoprotein cross reactively, whereas others are strain specific(Townsend et al., 1984; Townsend and Skehel, 1984). Protective effects of nucleoprotein specific cytotoxic T cell clones has not yet been demonstrated. There has been one report using genetically defined reassortant viruses in which cross reactive lysis was determined by the parental origin of the PB2 gene(Bennick et al., 1982). However, no cytotoxic T cell clones specific for PB2 have yet been identified. Similarly, cytotoxic T cell clones specific for M proteins have not yet been reported, though the M_2 protein has been shown to be expressed on the surface of infected cells(Lamb et

al., 1985).

In addition to cytotoxic T cells and neutralizing antibody, other phenomena have been demonstrated in response to influenza virus infection. Increased natural killer 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 reactions(Habershon et al., 1973) all have been demonstrated, but their contribution to recovery from infection or pathogenesis of disease is still controversial.

C. ANTIBODY DIVERSITY - GENERAL COMMENTS

The immune system has evolved elaborate diversity generating mechanisms to create an immune repertoire as diverse as the multiplicity of environmental antigenic stimuli to which it is exposed.. More specifically, the antibody repertoire can be accounted for in terms of the available variable region germ line genes and also diversity generating somatic events, respectively:

i. The initial antibody repertoire is determined by the multiple heavy and light chain variable region(V_H and V_L) gene segments and also the multiple joining gene segments[J_K(kappa), J(lambda), J_H and D] that reside in the germ line.

ii. During development these gene segments can join in various combinations and complete V gene products can randomly associate in different combinations(Weigert et al., 1978; Early et al., 1980). Somatic mutational mechanisms include variation at the sites at which gene segments join and the addition of nucleotides during gene segment joining(Alt and Baltimore, 1982), gene conversion(Dildrop et al., 1982) and point mutations(Weigert et al., 1970; reviewed by Tonegawa, 1983).

It is clear that the characterization of the molecular mechanisms of antibody diversity has been extensively investigated. Indeed, interests now are often directed to broader questions concerning the generation of antibody diversity. For instance, what is the relative importance of each of these distinct mechanisms? How do these molecular mechanisms account for changes in the quantity and quality of the the immune response as it progresses.

Recent work has suggested that the majority of the diversity may ultimately be due to somatic mechanisms. This interpretation may reflect accumulating evidence of somatically generated diversity(reviewed by Baltimore, 1981; Tonegawa, 1983) and also to the demonstration of an extremely high rate of mutation in immunoglobulin

variable region genes relative to non-immunoglobulin genes(Cook and Scharff, 1977; Kim et al., 1981; McKean et al., 1984). The strongest evidence, however, is derived from a careful quantitation of the total number of germ line gene segments and the combinational diversity that they may provide, in comparison to the estimated total diversity of the repertoire. This type of analysis has led Brodeur et al.(1984) to conclude that the major source of antibody diversity is somatic diversification.

It has long been known that immunoglobulin production during an immune response is a dynamic process. Both the quantity and quality of the response changes as it progresses. In particular, the variable regions of induced antibodies are altered. Both avidity for antigen(Eisen and Siskind, 1964) and the number of antigenic specificities(Heidelberger and Kendall, 1935) increase with time after immunization.

One view of the dynamics of this process sees the somatic evolution of V region structure as an antigen driven adaptive process of clonal selection that draws upon randomly generated V region diversity(Manser et al., 1985). This is a classical idea in immunology(Burnet, 1959) that is restated in terms of the current molecular understanding of somatic diversification. Idiotype regulation(Jerne, 1974), an alternative to this completely stochastic model, invokes regulation of the

levels of expression of specific V regions either before or during the course of the response irrespective of affinity of the antibody for the antigen.

1. APPROACHES TO ANTIBODY DIVERSITY IN THE INFLUENZA SYSTEM

a. IDIOTYPY

An idiotype, as originally defined by Oudin and Michel(1963) and Kunkel et al.(1963), is a collection of unique antigenic determinants(idiotopes) found on the variable region of antibody molecules from a single clone of antibody secreting cells. Therefore, a single paratope -- combining site -- would be associated with a single unique idiotype. Idiotypes which fit this definition have been designated individual idiotypes(IdIs). The finding of immunoglobulin molecules which share idiotypes necessitated a new designation -- the cross-reactive idiotype(IdX). While all idiotypes serve as phenotypic markers of V region genes, it is generally accepted that IdXs are the products of germ line genes whereas IdIs reflect a somatic mutational event(Greene et al., 1982). It is this role as a marker that makes idiotypes very useful in the study of antibody diversity.

The concept of an IdX and the dissociation between paratope and idiotype that it implies has been observed among a variety of immunoglobulin molecules. Evidence

has been obtained indicating that idiotypes may be shared among antibodies specific for different epitopes on the same antigen(Metzger et al., 1980; Liu et al., 1981), among antibodies specific for different antigens(Oudin and Cazenave, 1971) and even among immunoglobulins with unknown antigen specificity(Cazenave et al., 1974; Bona et al., 1979). Furthermore, anti-idiotype reagents have detected idiotypic determinants on somatic cells(Nepom et al., 1982) and T cells(Allison et al., 1982; Thomas et al., 1982; Infante et al., 1982). Though it is now known that the T cell receptor and immunoglobulins use completely different germ line genes, they are both members of the same supergene family and therefore are evolutionarily related(reviewed by Hood et al., 1985). In summary, the idiotype as a marker of V region genes provides a powerful tool for the study of diversity in the immune system, and it may be biologically relevant for more than just this system.

It is important in any introductory comments about idiotypy to point out its possible physiological relevance in terms of immune regulation and selection of the immunoglobulin receptor repertoire. It has been clearly demonstrated that lymphocytes expressing complementary receptors co-exist in the immune system(Bona et al., 1978; Cammisuli and Cosenza, 1980). At the level of B cells, anti-idiotypic antibodies can be

induced in animals known to possess B cell precursors expressing the corresponding idiotype(Eichmann, 1978). In addition, in the course of an immune response animals often produce anti-idiotypic antibodies against idiotypic determinants of their own antibodies, although mostly in low concentration(Kluskens and Kohler, 1974; Kelsoe and Cerny, 1979; Binion and Rodkey, 1982). At the level of T cells, idiotype-bearing and anti-idiotypic helper(Woodland and Cantor, 1978; Hetzelberger and Eichmann, 1978), suppressor(Bona and Paul et al., 1979; Weinberger et al., 1980) and effector T cells mediating delayed type hypersensitivity, cytotoxicity and contact sensitivity(reviewed by Bona, 1981) reactions can be induced in animals that express similar idiotypes and anti-idiotypes at the B cell level.

Jerne(1974) proposed a theory which considered the immune system as an idiotypic network. In his view the co-existence of idiotypes and anti-idiotypes is an essential feature of the immune system. Cells with complementary binding sites interact with each other and an equilibrium of idiotypes and anti-idiotypes is established. Immune responses are seen as disturbances of this equilibrium and attempts of the system to re-equilibrate. In addition, idiotypic interactions might be essential for generation of the functional receptor repertoire by acting as a selection mechanism.

b. V_L(KAPPA) GENES

Mouse V_K regions were initially divided into 31 groups based on protein sequence homology in the first framework region(residues 1-23) of BALB/c myeloma light chains. A group includes all K chains that differ by no more than 3 residues in this region(Potter, 1977). The V_K21 group has been most extensively studied. Complete V_L region sequences from 45 V_K21^+ plasmacytomas has divided the V_K21 group into 3 subgroups, each subgroup being defined as a set of V_K21 chains that share certain amino acid residues between positions 1 and 96(Weigert et al., 1978; McKean and Potter, 1979). Though it was long thought that prototype sequences for a subgroup represented a particular germ line gene, only recently has sequencing analysis of germ line V_K21 DNA segments shown that each subgroup is defined by a single germ line segment(Heinrich et al., 1984).

This group of light chains is of particular interest for the studies reported here because they are used in the anti-influenza response in disproportion to their expression in normal sera. While approximately 8.6% of normal BALB/c K-bearing serum immunoglobulin utilizes the V_K21 group of light chains(Julius et al., 1981), Staudt and Gerhard(1983) and Moran et al.(manuscript in preparation) have found that 22%(sample size=123) and 47%(sample size=20), respectively, of their anti-

influenza monoclonal antibodies are V_K21^+.

c. V_H GENES

Recently, mouse V_H genes have been grouped into 7 families on the basis of protein and DNA sequence(Dildrop, 1984), and also Southern blot analysis of genomic DNA(Brodeur and Riblet, 1984; Brodeur et al., 1984). At the nucleotide level $\geq$85% homology exists among the members of a family, while less than 70% homology is seen between families.

Analyses with recombinant inbred strains have indicated that the gene families lie genetically linked on chromosome 12. Their order is the following: centromere - V_HJ558 - V_HS107 - V_HQ52 - V_H7183 - 3'. The V_H J606 and 36-60 families tentatively lie centromeric to the J558 family, while mapping studies are still underway with the 441-4 family(Brodeur et al., 1984). Based on hybridizing Eco RI fragments, families contain as few as 2 individual germ line genes(V_H441-4) to as many as 50-60 members(V_HJ558). The total IgH-V locus has been estimated to contain approximately 100 germ line genes(Brodeur and Riblet, 1984).

The cDNA probes that define a V_H gene family offer a powerful tool for studying the antibody diversity of a particular immune response by slot blot hybridization analyses. These should allow technically less demanding quantitative analyses of antibody diversity at the

fundamental genetic level.

2. THE ANTI-INFLUENZA B CELL REPERTOIRE

The antibody response to glycoproteins expressed on the surface of influenza virus is extremely heterogeneous, involving responses to topographically distinct regions and to antigenic variants(Gerhard et al., 1978; Cancro et al., 1979). Recently, Staudt and Gerhard(1983) estimated that the repertoire of BALB/c antibodies directed to the H1 hemagglutinin of influenza A/PR/8/34 virus consists of at least 1500 different paratypes. If we consider that there are several other influenza virus proteins and hemagglutinin subtypes, and also the inherent limitations of discriminating paratypes on a panel of variant viruses, the size of the repertoire capable of interacting with influenza viruses is quite extensive.

The study of influenza specific monoclonal antibodies in this laboratory to date has repeatedly shown extensive cross reactive idiotypy(Liu et al., 1981; Liu et al., 1983; Moran et al., 1984). This was initially surprising in light of the extreme paratypic diversity that has been demonstrated in this response and also the classical idea of an idiotype as a private marker of a single B cell clone(Kunkel et al., 1963).

Analysis of the idiotypes of 3 PR8 hemagglutinin specific monoclonal antibodies -- P8, P20 and P28(all

directed to different determinants on the hemagglutin) -- with syngeneic anti-idiotype antibodies revealed the sharing of an idiotype as assessed both by HI assay and a competitive RIA. This finding of cross reactivity among antibodies with different antigenic specificities and different IgG subclasses confirms that they are the products of distinct B cell clones and suggests that the IdX is encoded by a germ line V region gene(Liu et al., 1981).

The study of the idiotypes expressed by monoclonal antibodies specific for B/Lee hemagglutinin -- B109, B118, B123, B141, B142 and B147 -- demonstrated that these monoclonal antibodies bear 3 categories of idiotypic determinants(Liu et al., 1981):

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

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

iii. IdXs borne by monoclonal antibodies specific for the same or overlapping antigenic determinants on B/Lee hemagglutinin.

It should be emphasized that the B/Lee specific monoclonal antibodies are of different subclasses. Moreover, although they are directed to overlapping

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 clone.

Recent work has uncovered a variety of very interesting idiotypic relationships(Moran et al., 1984). In particular, PY206 -- a monoclonal antibody obtained from a fusion following PR8 immunization which reacts with X-31 hemagglutinin(H3) but not with PR8 hemagglutinin(H1) -- bears 2 IdXs:

i. an IdX expressed by P20 and PY211. These 2 monoclonal antibodies are specific for different determinants of PR8 hemagglutinin. Most significantly, it is the first time that an IdX has been seen among monoclonal antibodies directed to hemagglutinins of different subtypes. It is noteworthy that in this case an IdX is shared among monoclonal antibodies from 3 different fusions.

ii. an IdX expressed by XY101, XY102 and XY108, monoclonal antibodies specific for X-31 hemagglutinin. Again, an IdX is shared among monoclonal antibodies from 2 distinct fusions.

The demonstration of shared idiotypes among monoclonal antibodies to both the H3 and H1 hemagglutinins with

syngeneic anti-PY206 idiotype antibodies has also been seen with monoclonal reagents(Moran et al., manuscript in preparation). SN3-9A and SP3-5A are monoclonal anti-PY206 idiotope antibodies that detect an IdX among H3 and H1 specific monoclonal antibodies in a sandwich assay.

We have attempted to gain some insight into the molecular nature of the PY206 cross reactive idiotopes by examining their $V_L(V_K21)$ and V_H gene families. Among the monoclonal antibodies bearing a cross reactive PY206 idiotope -- PY206, XY101 and XY102 -- PY206 is V_K21^-, while XY101 and XY102 use V_K21 subgroups A, D, E or F. Further evidence for the light chain independence of these idiotopes comes from studies with lambda light chain bearing monoclonal antibodies to nitrophenyl acetate and alpha-1-3-dextran. These antibodies are strongly IdX^+ positive when examined in both a sandwich and competitive RIA by SN3-9A.

We investigated V_H gene expression in these hybridomas by RNA slot blot hybridization analysis. PY206 and XY102 both use a V_H gene from the J558 family, while another strongly IdX^+ hybridoma -- XY101 -- derives it V_H gene from the 7183 family. The heavy chains encoded by these genes have very different sequences(Brodeur and Riblet, 1984; Dildrop, 1984) and there are no evident structural similarities to account for the idiotypic cross reactivity. Therefore, these

data suggest that these cross reactive idiotopes are not strictly dependent on the expression of particular V_H or V_L genes.

In summary, sharing of idiotypes among influenza specific monoclonal antibodies of different specificities and from different individual animals has been repeatedly seen in this laboratory. This has been observed with both polyclonal and monoclonal syngeneic anti-idiotype reagents. There are 2 general approaches to explaining these phenomena:

i. Antibody responses to a spectrum of influenza virus antigenic determinants may employ a relatively small portion of the total repertoire of germ line genes. Subsequent to somatic rearrangements and/or mutational events and clonal expansion induced by antigen, antibodies of different specificities are produced which bear idiotypes encoded by conserved segments of DNA.

ii. Clones expressing IdXs may be preferentially activated by regulatory T cells. This may be due to regulatory idiotypes(Bona et al., 1981). Actually, McNamara et al.(1983) have shown that idiotype specific T cells are able to recognize regulatory idiotypes on MOPC167 and TEPC15, phosphorylcholine binding antibodies. No

polyclonal or monoclonal anti-idiotype antibodies able to react with these shared structures have yet been obtained.

Clearly, the 2 hypotheses are not mutually exclusive.

Other workers have also studied the idiotypy of PR8 hemagglutinin-specific monoclonal antibodies. Staudt and Gerhard(1983) examined 13 monoclonal antibodies derived from a single mouse(#36). Ten of those 13 were directed to one of the 4 antigenic regions of the hemagglutinin. Of those 10, 4 were shown to share an idiotype, and no other monoclonal antibodies from their total panel of 125 bore this IdX. The reactivity patterns of these 4 idiotypically related antibodies were clearly distinguishable on a panel of variant viruses. From a second mouse(#37) they obtained 54 monoclonal antibodies. These were also largely directed to a particular antigenic region. Of the 54 monoclonal antibodies, 27(comprising 18 distinct reactivity patterns) shared an idiotype. When the remaining panel of monoclonal antibodies was examined, only 1 antibody from a different mouse shared the idiotype.

These results are quite similar to ours in that idiotypes are often found among monoclonal antibodies that are paratypically different. However, their results differ from those obtained in this laboratory in that they seldom observed idiotypes shared among antibodies

from different animals. A possible explanation for this difference is purely technical. We attempt to make our competitive RIA systems as sensitive as possible by examining the inhibition of binding to anti-idiotype of a labeled antibody other than the one employed to make the anti-idiotype reagent. This generally represents a lower avidity interaction of the idiotype and anti-idiotype that is more sensitive to inhibition. It also minimizes the role of individual idiotypes(IdIs) in this system. Staudt and Gerhard(1983), however, examine the inhibition of binding to anti-idiotype of a labeled idiotype that is the same as or paratypically very similar to the antibody used to generate the anti-idiotype antibodies.

Clarke et al.(1985) expanded upon the work of Staudt and Gerhard(1983) by restricting their study to monoclonal antibodies that are directed to the same antigenic region on the hemagglutinin and also that use the V_K21C subgroup. They further documented the clonal relationship of their monoclonal antibodies from a single mouse on the basis of variable region sequences and the characterization of unproductive rearrangements of germ line genes. Comparison of such clonally related monoclonal antibodies from a single mouse would allow analysis of the somatic events that contribute to intraclonal diversity. Interclonal comparisons of monoclonal antibodies from the same and different animals

would measure the contribution of combinatorial joining, junctional diversity and combinatorial association(V_H, V_K) to diversity in this response.

Clarke et al.(1985) drew the following interesting conclusions. Although combinatorial joining and association contribute to sequence diversity, they do not appear to have much effect on the fine specificity of the antibodies. On the other hand, somatic mutation contributed both to sequence diversity and differences in the fine specificity of antibodies. On the basis of intraclonal comparisons they estimated the somatic mutation rate to be extremely high -- at least 10^{-3} mutations per base pair per division. Interestingly, it appeared that amino acid replacement mutations in the light chain but not the heavy chain are selected for by antigen.

We adopted an approach in some ways similar to that of Clarke et al.(1985). However, rather than starting with a panel of monoclonal antibodies that we knew to be paratypically related, we sequentially selected variant viruses and prepared monoclonal antibodies specific for the variant but not for the parent virus. This allows us to examine a panel of monoclonal antibodies that are directed to PR8 hemagglutinins that generally differ by a single amino acid. This investigation is reported in the Results section.

D. ANTI-IDIOTYPE IMMUNIZATION

The immune network network hypothesis of Jerne(1974) suggests that each paratope recognizes an idiotope on another antibody. It is therefore probable that antigenic determinants borne by molecules other than antibodies(epitopes) must cross react with idiotopes. Consequently, idiotopes may represent internal images of nominal antigens, and the antigen repertoire may be largely reflected in the V gene products(idiotopes). A corollary of this theory is that anti-idiotypic antibodies or regulatory T cells can, like antigens, represent a force which can shape the immune repertoire.

The anti-idiotype has been conceptually subdivided into two categories that are important for an understanding of the possible mechanisms by which an anti-idiotype may elicit an immune response. The first type(beta) binds to the idiotype because the anti-idiotype bears a conformational structure similar to nominal antigen -- the classical internal image. It may stimulate a diverse population of clones(both idiotype$^+$ and idiotype$^-$) in a manner exactly analogous to that of nominal antigen. The second type(alpha), though lacking a conformational structure similar to antigen, interacts with idiotypic determinants on the antibody that is specific for nominal antigen. It will induce expansion, differentiation and maturation of clones of diverse

specificities expressing the corresponding idiotype.

Our attempts to elicit an influenza specific response by immunization with anti-idiotype has precedent in 5 viral systems -- hepatitis B virus(Kennedy and Dreesman, 1984), reovirus(Sharpe et al., 1984), rabies virus(Reagan et al., 1983), Sendai virus (Ertl and Finberg, 1984) Venezuelan equine encephalomyelitis virus(Roehring et al., 1984) and poliovirus(Fons et al., 1985). Our demonstration of cross reactive idiotypes on antibodies to hemagglutinins of different subtypes offers the theoretical possibility of using anti-idiotypes to elicit highly cross reactive anti-influenza responses.

E. ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT

CBA/N mice bear an X-linked defect(*xid*) that interferes with the development of a B cell subset which displays the Lyb5 surface antigen(Scher, 1982). They do not respond to thymus-independent type 2(TI-2) antigens such as trinitrophenylated(TNP)-Ficoll(Scher et al., 1975; Mosier et al., 1977). While anti-idiotype antibodies conjugated to Sepharose beads can stimulate proliferation of cells from these mice, soluble anti-immunoglobulin M(IgM) or anti-IgD will not(Sieckmann et al., 1978). They also have low ratios of membrane IgD to membrane IgM(Finkelman et al., 1975).

C_3H/HeJ mice possess a mutant gene(Lps^d), localized

to chromosome 4(Watson et al., 1978), which produces an unresponsiveness to both the immunological and pharmacological effects of endotoxin(Watson and Riblet, 1975).

A C_3H/HeJ congenic strain bearing the *xid* defect, C_3J.*xid*(Bona et al., 1980; Mond et al., 1983), has been developed. Interestingly , this strain bears all the defects of the parental CBA/N and C_3H/HeJ strains in an enhanced form and also additional defects. Unlike cells of the parental strains, lymphocytes from these mice are unresponsive *in vitro* to thymus-independent type 1(TI-1) antigens such as TNP-*Brucella abortus*(TNP-BA). Several B cell mitogens do not stimulate these cells, nor do anti-immunoglobulin antibodies conjugated to Sepharose beads. They are also unresponsive to certain T cell derived helper factors.

The antigen used in the present study was the influenza A virus, A/PR8/34(H1N1)(PR8 virus). Previously, it had been reported that mice bearing the *xid* defect did not make a detectable hemagglutination-inhibiting(HI) antibody response after immunization with Formalin-inactivated influenza virus(Lucas et al., 1978). Here we provide evidence that mice bearing the *xid* defect develop adequate antibody responses to influenza virus antigens. However, significant differences in the relative amounts of different isotypes were observed in

comparison to the responses of strains lacking this defect.

VII. MATERIALS AND METHODS

A. ANIMALS

Six to 12 week old BALB/c mice and New Zealand White rabbits were obtained from Jackson Laboratories(Bar Harbor, Me.) C_3H/HeJ and C_3HeB/FeJ mice were obtained from Jackson Laboratories(Bar Harbor, Me.). C_3H/HeN mice were a gift from Dr. J. Mond(Uniformed Services University of the Health Sciences, Bethesda, Md.). C_3J.*xid* mice were obtained from Dr. W.E. Paul(National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

B. VIRUSES

The following influenza viruses were used in these studies: B/Lee/40(B/Lee virus), A/PR/8/34(H1N1)(PR8 virus), A/Houston/18515/84(H1N1), A/Singapore/1/57(H2N2), X-31(H3N2)(X-31 virus) -- a recombinant virus A/Aichi/2/68(H3N2) x A/PR/8/34(Kilbourne et al., 1971), A/Chicken(Scotland)/59(H5N1), PY102-V1 and VM113-V1. The last 2 viruses are laboratory selected variants of PR8 virus.

All viruses were grown in the allantoic cavity of 10 to 11 day old embryonated chicken eggs. Allantoic fluid virus was used in HI antibody titrations and neutralization assays. Virus purified on 30-60% sucrose gradients according to standard procedures (Palese and Schulman, 1974) was used in RIAs. All viruses were

stored at -70 degrees centigrade.

C. PREPARATION OF ANTIBODIES.

1. BALB/c INFLUENZA SPECIFIC MONOCLONAL ANTIBODIES

Hybridoma cell lines producing monoclonal antibodies against the hemagglutinin of influenza A viruses PY102-V1 and VM113-V1 were prepared by fusion of spleen cells from immunized BALB/c mice with SP2/0 myeloma cells(Kohler and Milstein, 1976; Koprowski et al., 1977). Briefly, BALB/c mice were primed 1 to 3 months earlier by intraperitoneal injection of purified intact virus(10 micrograms viral protein/mouse) and were boosted 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 cells:myeloma cell) in 50% PEG[polyethylene glycol, MW 4000, Art. 9727(3194408), American Scientific Products, Edison, N.J.]. The selective HAT medium(1 x 10^{-4} M hypoxanthine, 3 x 10^{-7} M aminopterin and 1.6 x 10^{-3} M thymidine, all from Sigma Chemical Co., St. Louis, Mo.) 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 RIA. Monoclonal antibodies specific for egg carbohydrates, determined by showing that the supernatant fluid also bound to plates coated with an influenza B virus, were eliminated.

H36-5-3 and H36-7-3 ascites were purified on a PR8

virus-Sepharose column. The remaining monoclonal antibodies were initially ammonium sulfate precipitated or purified on a Sephacryl S-300 column. Antibodies of the IgG3, IgG2b and IgG2a isotypes were further purified on a protein A-Sepharose column. IgG1 antibodies were further purified by ion exchange chromatography.

2. HUMAN INFLUENZA SPECIFIC MONOCLONAL ANTIBODIES

Human influenza specific monoclonal antibodies, derived from Epstein-Barr virus transformed peripheral blood lymphocytes, were a gift from Dr. Nolan Sigal(Merck and Co., Inc., Rahway, N.J.)

3. SYNGENEIC MONOCLONAL ANTI-IDIOTOPE ANTIBODIES

Syngeneic monoclonal anti-idiotope antibodies were prepared to PY206(SN3-1A, SN3-9A and SP3-5A) and PY211(63-4) by T. Moran(Mt. Sinai School of Medicine, Department of Microbiology, New York, N.Y.). PY206 is a monoclonal antibody specific for the H3 hemagglutinin of X-31 virus, while PY211 is a monoclonal antibody specific for the H1 hemagglutinin of PR8 virus.

The immunization scheme was different than that used for the production of virus specific monoclonal antibodies. Briefly, BALB/c mice received 100 micrograms of PY206 or PY211, coupled to KLH and emulsified in FCA, intraperitoneally and also subcutaneously in the footpads and inguinal lymph node region. After a 2 week interval the mice were immunized weekly for 3 weeks in a manner

identical to the initial immunization, except that the monoclonal antibody-KLH conjugate was emulsified in FIA. After a 1 week interval the final immunization was performed. This differed from the previous 3 immunizations in that the monoclonal antibody was not necessarily coupled to KLH and an intravenous injection was included. The fusion was done 3 days later as described above for the production of influenza specific monoclonal antibodies. The monoclonal anti-idiotope antibodies were purified on the appropriate PY206 or PY211-Sepharose column.

All hybridoma cell lines were suspended in 5% DMSO-95% FCS and stored in a liquid nitrogen tank(Cryo-med Co., Mt. Clemens, Mi.) The monoclonal antibodies used in the studies reported in the Results section, along with their isotype and specificity, are summarized in Table II.

4. HETEROLOGOUS ANTI-IDIOTYPE ANTIBODIES

New Zealand White rabbits(Jackson Laboratories, Bar Harbor, Me.) were primed intradermally with 0.5-1.0 mg of purified monoclonal antibody(PY102, VM113 and VM202) in Freund's complete adjuvant. After three weeks they were boosted(injection identical to that used in priming) and then bled 10 days later. Sera from rabbits immunized with PY102, VM113 or VM201 were adsorbed to affinity columns in which the antibody coupled to Sepharose was

Table II. Summary of monoclonal antibodies

MAb	Fusion #	Isotype	Specificity
PY102	1	G1	H1[a]
VM113	2	G2a	H1
VM201	3	G1	H1
VM202	3	G3	H1
H36-5-3[b]	4	G3	H1
H36-7-3[b]	4	G3	H1
HPCG15[c]	5	ND	PC
PT109	6	G2a	H1(HA-2)[d]
XY101	7	G2a	H3
VM301	8	G2b	H1
VM303	8	G1	H1
VM310	8	G2a	H1
PY211	9	G2b	H1
PY206	9	G2b	H3
B109	10	G1	B
BY104	11	G2a	B
SN3-1A	12	G1	PY206 Id[e]
SN3-9A	12	G1	PY206 Id
SP3-5A	12	G1	PY206 Id
63-4	13	G1	PY211 Id

Table II(cont.).

[a]Hemagglutinin subtype

[b]A gift from Dr. W. Gerhard(Wistar Institute, Philadelphia, Pa.)

[c]A gift from Dr. P. Gearhart(Johns Hopkins U., School of Hygiene and Public Health, Baltimore, Md.) This antibody is representative of all PC(phosphorylcholine) binding monoclonal antibodies used in these studies(see Table X).

[d]Carboxy terminal cleavage product of hemagglutinin

[e]PY206 idiotope

VM202, VM113 or VM201, respectively. Anti-idiotype antibody was eluted from the column with 0.1M glycine-HCl buffer(pH 2.5). The eluate was then passed over a BALB/c normal serum immunoglobulin-Sepharose column to remove anti-isotype and anti-allotype antibodies. The adsorptions were repeated until there was no detectable binding of antibody to the column.

D. ANTIBODY PURIFICATION

Several methods of protein purification were used. Generally, an unpurified antibody sample was initially ammonium sulfate precipitated or purified by gel filtration. It was then affinity purified on an antigen-Sepharose or Protein A-Sepharose column, or it underwent an ion exchange purification.

1. AMMONIUM SULFATE PRECIPITATION(reviewed by Heide and Schwick, 1978).

Antibodies were precipitated from solution by slowly adding saturated ammonium sulfate until a 40% solution was created. After an overnight incubation at 4 degrees centigrade, the precipitate was resuspended in an appropriate buffer and then extensively dialyzed against that buffer.

2. GEL FILTRATION(Belew et al., 1978)

As much as 10 ml of ascitic fluid was applied to a gel filtration column packed with Sephacryl S-300(superfine)(Pharmacia Fine Chemicals, Piscataway,

N.J.) in a 0.02 M Tris buffer(pH 8, 0.3% sodium azide). The column(C26/100 - Pharmacia Fine Chemicals, Piscataway, N.J.) is 100 cm long with an internal diameter of 26 mm. It was stored and all filtrations were done at 4 degrees centigrade. Ascitic fluid was pumped upward through the column at a constant flow rate and 3-4 ml fractions were collected. Both the pump and fraction collector are made by LKB Inc.(Bromma, Sweden).

3. PROTEIN A PURIFICATION(Ey et al., 1978)

A protein A-Sepharose conjugate was purchased from Pharmacia Fine Chemicals(Piscataway, N.J.), swollen in a 0.1 M phosphate buffer(0.02 % sodium azide) and packed in a column. The pH of all antibody preparations was adjusted to ~8 before application to the column. Different isotypes were eluted with a 0.1 M citrate buffer adjusted to different pHs. IgG3 and IgG2a antibodies were eluted at pH 4.5, while IgG2b antibodies eluted at pH 3.5. Fractions(3-4 ml) were collected in tubes containing 0.5 ml of 1 M Tris-HCl buffer(pH 8.5) to avoid denaturation of the antibody. The column was stripped of any residual bound protein with a 0.1 M glycine-HCl buffer(pH 2.5), re-equilibrated in the 0.1 M phosphate buffer(0.02% sodium azide) and stored at 4 degrees centigrade.

4. ANTIGEN SPECIFIC AFFINITY PURIFICATION(Lecomte and Tyrrel, 1976)

Either a purified virus or monoclonal antibody was coupled to cyanogen bromide activated Sepharose 4B(Pharmacia Fine Chemicals, Piscataway, N.J.). In order to expose internal viral proteins on the column, the virus was treated with a detergent prior to the coupling reaction. Briefly, the virus was 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 then resuspended in 0.9% NaCl containing 10% octoglucoside(Sigma Chemical, St. Louis, Mo.) for 30 minutes at 37 degrees centigrade.

The actual coupling of viral protein or immunoglobulin to the activated Sepharose was achieved by an overnight incubation at 4 degrees centigrade in a 0.1 M bicarbonate buffer(pH 8.3). The remaining active groups on the Sepharose were blocked by 1 M ethanolamine in borate buffer(pH 8). The absorbent was then washed alternately with low pH(acetate buffer, 0.1 M, pH 4) and high pH(borate buffer, 0.25 M, pH 8) buffers for 3 cycles to remove uncoupled viral proteins and blocking agent.

Virus specific monoclonal antibodies or rabbit-anti-idiotype sera were purified by adsorption to the appropriate column followed by elution with a 0.1 M glycine-HCl buffer(pH 2.5). The eluted proteins were collected in 3-4 ml fractions into tubes containing 0.5 ml of 1 M Tris-HCl buffer(pH 8.5). The columns were re-

equilibrated with several column volumes of a 0.1 M phosphate buffer(pH 8, 0.02% sodium azide) and stored at 4 degrees centigrade in this buffer.

5. ION EXCHANGE PURIFICATION(reviewed by Fahey and Terry, 1978)

Monoclonal antibodies, primarily of the IgG1 isotype, were purified by adsorption to DEAE-cellulose(Whatman Biochemicals Ltd., Maidstone, Kent), an anion exchanger. The unpurified antibody, which had been extensively dialyzed against a low salt buffer(0.005 M phosphate buffer, pH 8), was applied to the column. The column contained approximately 0.07 ml swollen DEAE-cellulose/mg protein applied to the column. The adsorbed protein was eluted by an ionic strength gradient(0.005-0.1 M phosphate buffer, pH 8). Fractions of 3-4 ml were collected and the IgG was found in the first peak(as determined by OD_{280}) eluted from the column.

E. CONCENTRATION OF PURIFIED PROTEINS

Purified proteins were concentrated in an Amicon ultrafiltration system(Lexington, Ma.) under nitrogen pressures $\leq$70 psi(4.7 atm). The pore size of the filters was such that molecules with molecular weights greater than 10,000 were excluded(Diaflo Ultrafilters, PM10, Amicon Corp., Lexington, Ma.).

F. DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration of purified antibodies and

viruses was determined by the method of Lowry et al.(1951).

G. SEROLOGICAL ASSAYS

Samples were sera, ascites, supernatants or in a purified form. They will be referred to as "antibodies" below.

1. HEMAGGLUTINATION INHIBITING(HI) ANTIBODY TITRATION

Antibodies were treated with *Vibrio cholerae* receptor-destroying enzyme(RDE)(Centers for Disease Control, Atlanta, Ga.) (Dowdle et al., 1979) before assay of HI titer. Tests were performed in microtiter plates with 0.25% human type O erythrocytes and 3 hemagglutinating units of virus according to a standard procedure(Hierholzer and Sugg, 1969). The $-\log_2$ of the highest dilution at which hemagglutination was inhibited was the HI titer of the antibody.

2. NEUTRALIZATION ASSAYS

Both pre-inoculation and post-inoculation neutralization titers were determined in the following way: Madin-Darby canine kidney cell monolayers were infected with either virus or a mixture of virus and antibody. After a 1 hour adsorption period the inoculum was removed and 5 ml of agar overlay containing 1x MEM(minimum essential medium, Gibco, Grand Island, N.Y.), 0.5% agar and 2 micrograms/ml trypsin was added. The number of plaques was estimated after 3-4 days of

incubation at 37 degrees centigrade in an atmosphere of 5% CO_2.

a. PRE-INOCULATION NEUTRALIZATION ASSAY

In this assay(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 antibodies were mixed and allowed to incubate at 37 degrees centigrade for 45 minutes. At the end of the reaction period, 0.2 ml of the mixtures was inoculated onto Madin-Darby canine kidney cell monolayers. After a 1 hour period of adsorption, the inoculum was removed, the dishes were washed with PBS, and 5 ml of agar overlay was added. The number of plaques was counted after 3-4 days of incubation.

b. POST-INOCULATION NEUTRALIZATION ASSAY

In this assay(Jahiel and Kilbourne, 1966) the Madin-Darby canine kidney cell monolayers were infected with approximately 100 PFU of virus. After virus adsorption an agar overlay containing serial dilutions of RDE treated antibodies were added, and plaques were counted after 3-4 days incubation.

The reciprocal of the antibody dilution at which 50% plaque reduction occurred was the pre-inoculation or post-inoculation neutralization titer.

c. SELECTION OF VIRUS VARIANTS

Viruses were grown on Madin-Darby canine kidney cells in the presence of an excess of RDE-treated antibodies. Plaque-picked viruses were confirmed as variants by HI or neutralization assay with the monoclonal antibody used in selection.

3. RADIOIMMUNOASSAYS(RIAs)

a. VIRUS BINDING ASSAY(INCLUDING ISOTYPE DETERMINATION)

Binding assays to detect and quantitate antibodies specific for a particular virus and also the isotype of these antibodies were routinely done. Assays to detect virus binding antibodies, regardless of isotype, were most widely used to screen hybridoma supernatants, while assays to determine the isotypes of virus binding antibodies were used to their greatest extent in studies of the anti-influenza response in mice bearing the _xid_ defect.

Briefly, 96 well polyvinyl chloride plates(Fisher Scientific, Springfield, N.J.) were coated with virus(50 microliters of purified virus, 50 micrograms/ml) for 90 minutes at 37 degrees centigrade(or overnight at 4 degrees centigrade) and saturated with 0.02 M borate buffer(pH 8)-1% BSA for 90 minutes at 37 degrees centigrade. After 3 washings with PBS, 50 microliters of antibody was added and incubated for 90 minutes at 37 degrees centigrade. The presence of bound antibody was then determined by applying 50 microliters of an ^{125}I-

anti-mouse Ig reagent(50,000 cpm). This was generally an ^{125}I-goat-anti-mouse Ig reagent(New England Nuclear, Boston, MA.) or an ^{125}I-rat-anti-mouse kappa light chain reagent. For isotype determinations ^{3}H-goat-anti-mouse isotype antibodies(a gift from Dr. P. Mongini, Hospital for Joint Diseases, New York, N.Y.) were used. After incubation for 90 minutes at 37 degrees centigrade , the wells were extensively washed(15 times with PBS), then cut and counted in a Beckman gamma-counter(Beckman Co., Palo Alto, CA) for assays in which ^{125}I was used or scintillation counter for assays in which ^{3}H was used.

There are further points that should be noted concerning the isotype determinations done in the study of mice bearing the *xid* defect. Included with the various serum samples of unknown isotype content were PR8 hemagglutinin specific monoclonal antibodies of known isotype. Application of these monoclonal antibodies at various concentrations provided standard curves for each isotype(Figure 1). IgM, IgG3, IgG1, IgG2b and IgG2a PR8 hemagglutinin specific monoclonal antibodies were available in our laboratory. The PR8 hemagglutinin specific IgA monoclonal antibody was a gift from Dr. Walter Gerhard(Wistar Institute, Philadelphia, Pa.). These standard monoclonal antibodies were purified on a PR8-Sepharose column. Their total protein content was then determined. The specificity of these reagents has

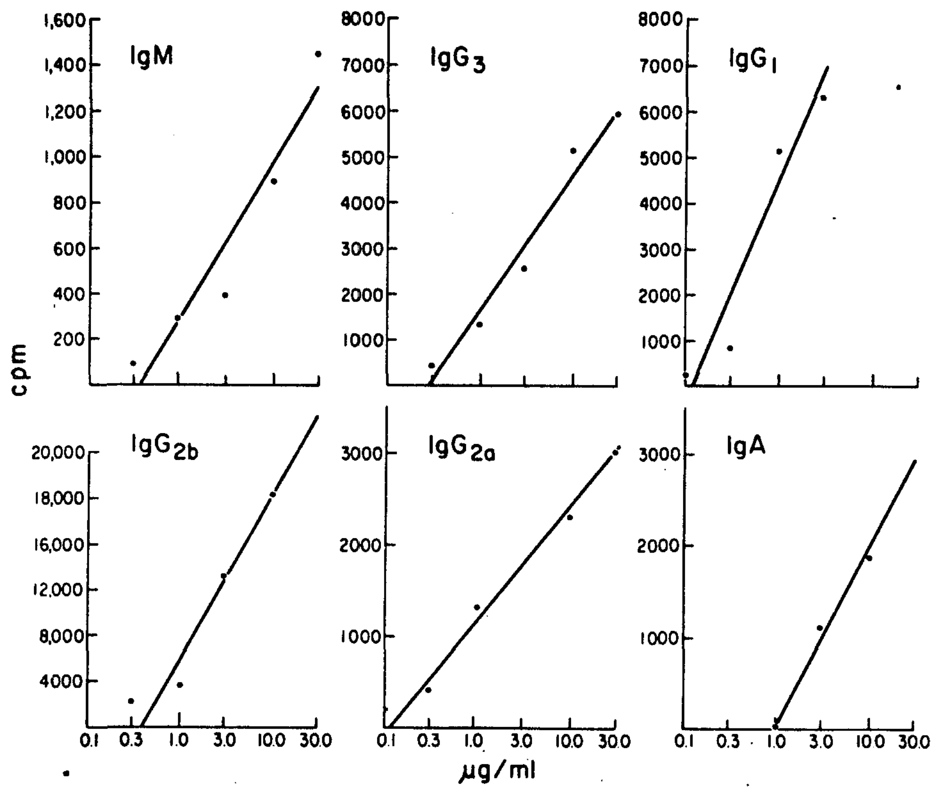

Figure 1. Standard curves generated with affinity purified, PR8 virus specific monoclonal antibodies of known isotypes. Each point represents the average of triplicate or quadruplicate determinations. Lines have been fitted by a linear regression analysis.

been documented repeatedly in our laboratory(unpublished data) and in published reports from other laboratories(Mongini et al., 1981; Stein et al., 1983).

Also, in the study of mice bearing the xid defect PR8 virus specific antibody of particular isotypes in the sera of mice after immunization was determined by using pools of sera of 5 mice in a similar fashion. Concentrations of isotype specific anti-influenza antibodies were determined from the standard curves shown in Figure 1. Concentrations of anti-influenza antibodies of particular isotypes in the sera of mice after infection were obtained from individual sera(5 mice/group). Background binding of sera from non-immune mice was subtracted from all values.

b. SANDWICH ASSAY

The sandwich assay provided a very sensitive means of screening large numbers of monoclonal antibodies for particular idiotypic determinants.

Briefly, microtiter plates were coated with 50 microliters of particular monoclonal antibodies at several concentrations(generally 20, 10, 5 and 2.5 micrograms/ml) for 90 minutes at 37 degrees centigrade and then saturated with 0.02 M borate buffer(pH 8)-1% BSA. After 3 washes with PBS, 50 microliters of purified anti-idiotype antibodies(20 micrograms/ml) were added and incubated at 37 degrees centigrade for 90 minutes.

Another 3 washes with PBS were performed, and then 50 microliters(50,000 cpm) of a purified ^{125}I-labeled monoclonal antibody, often the same monoclonal antibody used to elicit the anti-idiotype antibodies used in the preceding step, was added and incubated for 90 minutes at 37 degrees centigrade. After 15 washes with PBS, the wells were cut and counted in a gamma counter.

c. COMPETITIVE ASSAY

This assay provided a highly specific, though less sensitive means of detecting idiotypic determinants or V_K21 light chains on particular antibodies. The rabbit antiserum designated M70E(Julius et al., 1981), specific for V_K21 subgroups A, D, E, F and to some extent C, and also the purified myeloma proteins PC2880(V_K21 A) and PC10916(V_K21 C) were gifts from Dr. M. Weigert(Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.)

Briefly, microtiter plates were coated with 50 microliters of an anti-idiotype reagent(generally 5 micrograms/ml) or the rabbit-anti-V_K21 antibodies(1:1000 dilution) for 90 minutes at 37 degrees centigrade and then saturated with 0.02 M borate buffer(pH 8)-1% BSA. After 3 washes with PBS, 50 microliters of a mixture of various dilutions of an antibody of interest and an ^{125}I-labeled antibody were added and incubated at 37 degrees centigrade for 90 minutes. The labeled antibody was

PC2880 in the V_K21 assay and an antibody known to bind to the given anti-idiotype reagent in the idiotype system. After 15 washes with PBS, the wells were cut and counted in a gamma counter. The percent reduction in counts bound, relative to the appropriate non-inhibitory control, was calculated.

H. IODINATION OF PURIFIED ANTIBODIES

Purified 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 by passage over a Sepharose G-25(fine grade) column.

I. ADSORPTION OF IMMUNE SERA

Microtiter plates were coated with 50 microliters of virus(100 micrograms/ml) or monoclonal anti-idiotope antibodies(50 micrograms/ml) for 90 minutes at 37 degrees centigrade(or overnight at 4 degrees centigrade) and saturated with 0.02 M borate buffer(pH 8)-1% BSA for another 90 minutes at 37 degrees centigrade. After these plates were washed 3 times with PBS, 50 microliters of various dilutions of a particular serum were added. The adsorption period was for 90 minutes at 37 degrees centigrade. The contents of this first adsorption plate were then replica plated on to a second adsorption plate

and incubated as before. Generally 3 adsorptions were performed. After the last adsorption the contents of the adsorption plate were replica plated on to an appropriately coated microtiter plate for radioimmunoassay.

J. IMMUNIZATIONS AND INFECTIONS

1. ATTEMPT TO ELICIT AN ANTI-INFLUENZA IMMUNE RESPONSE BY IMMUNIZATION WITH MONOCLONAL ANTI-IDIOTOPE ANTIBODIES

a. SERUM STUDIES

Sera from 2 different immunizations were analyzed for their expression of particular idiotopes. In the first immunization BALB/c mice were parenterally primed and boosted 30 days later with 10 micrograms of purified PR8(H1N1) or X-31(H3N2) virus. Individual sera from the secondary PR8(10 days post-boost) and X-31(15 days post-boost) immune responses were examined.

In the second case groups of 8 BALB/c mice were each parenterally primed and then boosted 30 days later with 10 micrograms of purified B/Lee, X-31, PR8, A/Singapore(H2N2), A/Houston(H1N1) or A/Chicken(H5N1) viruses. Bleedings were done on days 3, 10, 20, 33, 37 and 44. Individual sera were examined.

b. ANTI-IDIOTOPE IMMUNIZATION

BALB/c mice(5 animals/group) received 5, 1 or 0.1 micrograms of alum precipitated monoclonal anti-idiotopes(SN3-1A, SN3-9A, 63-4 or IDA-10)

intraperitoneally. IDA-10 is a monoclonal anti-idiotope specific for A48, a bacterial levan binding monoclonal antibody. They then received intraperitoneally 10 micrograms of X-31 or PR8 virus either 7 or 14 days after anti-idiotope priming. All mice were then bled on days 7, 14 and 21 after their respective virus immunization to determine whether anti-idiotope immunization primed for subsequent antiviral responses.

2. ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT

Five CBA/N, C_3H/HeJ, and C_3J.*xid* mice received an intraperitoneal injection of 10 micrograms of viral protein in 0.2 ml of saline on days 0 and 30. Orbital bleedings were done before the immunization of days 0 and 30 and also on days 5, 10, 30, 33, 40, 50 and 60. Pooled sera were employed in the solid phase radioimmunoassay isotype determination.

Five mice each from the CBA/N, C_3H/HeJ, C_3H/HeN and C_3HeB/FeJ strains underwent aerosol infection with a virus suspension containing 3×10^5 PFU/ml. Twenty-one days post-infection, mice were sacrificed and bled. Individual sera were used for the isotype determination.

K. DETERMINATION OF V_H GENE FAMILIES

1. PURIFICATION OF RNA

RNA was prepared from 10^8 cells obtained from logarithmically growing hybridoma cell cultures according

to the method of Glisin et al.(1974). The cells were washed 3 times with PBS and then resuspended in 5 ml of guanidine thiocyanate buffer. After 3 hours at room temperature(or overnight at 4 degrees centigrade) the cell suspension was passed 4 times through an 18 gauge needle. This suspension was layered over 2 ml of 5.7M CsCl and then spun for 17 hours at 30,000 rpm(20 degrees centigrade). After removing the supernatant the pellet was resuspended in double distilled water and then ethanol precipitated 3 times. The pellet was finally dissolved in a Tris-EDTA buffer and stored at -20 degrees centigrade. The RNA concentration was determined from the OD_{260}.

2. SLOT BLOTTING

Forty microliters of various concentrations(usually 5.0, 1.0 and 0.2 micrograms/ml) of the RNA, dissolved in 20X SSC buffer, was applied to BA35 nitrocellulose filters(Schleicher and Schuell, Keene, N.H.) with a Minifold II apparatus(Schleicher and Schuell, Keene, N.H.). Filters were air dried and then baked for 2 hours at 80 degrees centigrade under vacuum.

3. HYBRIDIZATION STUDIES

Hybridization of ^{32}P-nick translated cDNA probes, prototypes of 7 murine V_H families, and also a J_H probe to the blotted RNAs was performed by A. Manheimer(Dept. of Microbiology, Mt. Sinai School of Medicine) according

to the method of Brodeur and Riblet(1984).

Nick translation of the probes was performed as follows. In a 1.5 ml conical Eppendorf tube a mixture of 100 ng of the DNA to be labeled, 1 nmole of each unlabeled dNTP(dATP, dGTP and dTTP) , 100 pmoles of [alpha-^{32}P]dCTP, 20 microliters of 5x nick translation buffer[0.25 M Tris-HCl(pH 7.2), 0.05 M $MgSO_4$, 0.5 mM dithiothreitol and 500 micrograms/ml nuclease-free BSA] and double distilled H_2O(to bring the total volume to 100 microliters) was made. One microliter of DNase I(0.1 microgram/ml) was added and then the mixture was incubated for 15 minutes at 37 degrees centigrade. Five units(Richardson et al., 1964) of E. coli DNA polymerase I was added, the mixture was vortexed and then incubated for 60 minutes at 14 degrees centigrade. The reaction was stopped by the addition of 2 microliters of 0.5 M EDTA. The nick translated DNA was separated from the unincorporated dNTPs by passage over a Sephadex G-50(fine grade) column.

Filters on which the RNAs had been slot blotted underwent a 3 hour prehybridization period at 65 degrees centigrade in 10x Denhardt's solution, 5 mM EDTA, 50 micrograms/ml sonicated salmon sperm DNA, 10 micrograms/ml poly A, 0.1% SDS and 3x SSC buffer. The hybridization solution was the same as for the prehybridization with the addition of 10% dextran sulfate

and 10 ng/ml V_H probe nick translated with ^{32}P-dCTP to a specific activity of 100-200 cpm/pg.

VIII. RESULTS

A. DIVERSITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

1. GENERATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

A series of PR8 variant viruses and a corresponding set of specific monoclonal antibodies has been obtained. The experimental protocol is shown schematically in Figure 2. PY102, a monoclonal antibody directed to the H1 hemagglutinin of PR8 virus, was used in antibody excess to select a variant virus, PY102-V1. Immunization with PY102-V1 and the subsequent fusion resulted in VM113, a monoclonal antibody that binds to the variant(PY102-V1), but not to the parental PR8 virus. VM113 was then used to select variants of PY102-V1, one of which has been designated VM113-V1. VM201 and VM202 are monoclonal antibodies that were generated by immunization with VM113-V1. They bind to VM113-V1 but not to PY102-V1. It is important to note that previous analysis of such virus mutants by peptide mapping and sequencing of the cloned cDNA of the relevant hemagglutinin genes has generally shown that each contains a single amino acid substitution in one of the 4 major antigenic regions of the hemagglutinin(Caton et al., 1982; Webster et al., 1983).

It is noteworthy that the 9 virus variants of PY102-V1 selected in the presence of VM113 alone or in the

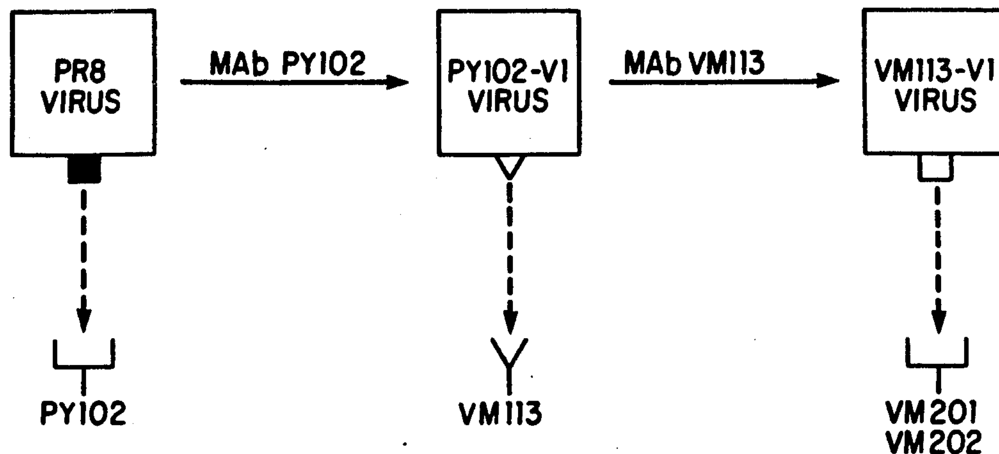

Figure 2. Scheme of generation of sequential virus variants and their corresponding monoclonal antibodies. A solid arrow beneath the name of a monoclonal antibody indicates selection of a virus variant with that particular monoclonal antibody. A broken arrow directed from a virus to a monoclonal antibody indicates that immunization with that virus and the subsequent fusion resulted in production of the particular monoclonal antibody.

presence of both VM113 and PY102 can be divided into 2 groups(Table III). One group of variant viruses -- represented by 4 of the 5 variants selected in the presence of VM113 alone -- is "PR8-like" in that these viruses are inhibited by PY102 in an HI assay. The remaining VM113 selected variant(VM113-V4) and all the variants selected in the presence of both VM113 and PY102 comprise the second group. These variant viruses are not inhibited by either PY102 or VM113 in the HI assay. We were not successful in producing a monoclonal antibody specific for a representative of this second group.

2. ANTIGEN SPECIFICITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

The reactivity of these monoclonal antibodies with the appropriate viruses is shown by hemagglutination inhibition assay in Table IV. The reciprocal specificities of VM113 and the 3 other monoclonal antibodies is evident. The hemagglutination inhibition titer of VM113 for PY102-V1 exceeds its titer for PR8 and VM113-V1 by 11 log_2 units. The titers of PY102, VM201 and VM202 for PR8 and VM113-V1 exceed their titers for PY102-V1 by at least 8 log_2 units.

These reactivity patterns were confirmed in a neutralization assay(Table V). The reciprocal specificities of VM113 and the 3 other monoclonal antibodies is again evident. While the neutralization

Table III. Characterization of variant viruses of PY102-V1 selected by VM113 - HI assay

Virus	MAbs Used in Selection	Reactivity with MAbs: PY102[a]	VM113
VM113-V1	VM113	12[b]	0
VM113-V2	VM113	11	0
VM113-V3	VM113	12	0
VM113-V4	VM113	0	0
VM113-V5	VM113	12	0
VM113-V6	VM113 & PY102	0	0
VM113-V8	VM113 & PY102	0	0
VM113-V9	VM113 & PY102	0	0
VM113-V10	VM113 & PY102	0	0
PR8	--	12	0
PY102-V1	PY102	4	12

[a]RDE-treated ascites

[b]$-\log_2$ of highest dilution at which hemagglutination is inhibited

Table IV. HI titers - specificity of monoclonal antibodies to sequential PR8 virus variants

	Monoclonal Antibody			
Virus	PY102	VM113	VM201	VM202[a]
PR8	12[b]	0	12	11
PY102-V1	4	11	0	0
VM113-V1	12	0	12	11

[a]RDE-treated ascites

[b]$-\log_2$ of highest dilution at which hemagglutination is inhibited

titer of VM113 for PY102-V1 exceeds its titer for PR8 or VM113-V1 by at least 1.5 $\log_{10}$ units, the titers of PY102, VM201 and VM202 for PR8 or VM113-V1 are at least 1.2 $\log_{10}$ units greater than their titers for PY102-V1. PR8 and VM113-V1 are indistinguishable with the antibody reagents presently available, and the possibility exists that VM113-V1 represents a revertant to PR8 virus. It is evident that sequence data will ultimately be necessary to determine if this is the case.

The data in Table IV and V do not distinguish the paratypes of PY102, VM201 and VM202. This problem was resolved by examining the reactivity patterns of these 3 monoclonal antibodies on a larger panel of variant viruses. Variants were selected by passage of each of 10 allantoic seed viruses obtained from plaque-picked populations of PR8 and PY102-V1 viruses in the presence of an excess of PY102, VM201 or VM202. Fifty-six variant viruses were obtained. In all but 3 instances variants selected in the presence of one monoclonal antibody were variants also with respect to both of the other antibodies. The 3 exceptional viruses(discussed further below), variants of PR8 virus selected in the presence of PY102, enabled us to distinguish the specificity of PY102 from those of VM201 and VM202. However, further selection of variants was required to distinguish the specificities of VM201 and VM202.

Table V. Neutralization assay - specificity of monoclonal antibodies to sequential PR8 virus variants

Virus	Monoclonal antibody			
	PY102	VM113	VM201	VM202
PR8	6.0	<3.0	5.1	4.8
PY102-V1	4.5	4.5	<3.6	<3.6
VM113-V1	6.0	<3.0	5.1	4.8

[a]RDE-treated ascites

[b]$-\log_{10}$ of antibody dilution at which a 50% reduction in plaque number is seen

PY102 selected variants from 2 different PR8 plaques have been examined in more detail. The results of an HI assay are shown in Tables VI and VII. The monoclonal antibodies PY202 and VM105 have been included because they are known to be directed to sites on the hemagglutinin that do not overlap with the site recognized by the sequential variant-specific monoclonal antibodies. The combining site of PY102 is shown to be different from that of VM201 and VM202 in Table VI(panel A) where it has greatly reduced inhibitory activity with a PY102 selected variant of PR8 virus plaque 2(PY102-VP2), yet VM201 and VM202 retain inhibitory activity with this variant. This is seen again in panel B with PY102-VP5. Variants of the parental PR8 viruses selected with VM201 or VM202 -- VM201-VP2, VM201-VP5, VM202-VP2 and VM202-VP5, respectively -- were not able to show this difference.

VM201 and VM202 could only be discriminated by PY102 selected variants of PR8 that were further selected with VM201 or VM202(Table VII). The most definitive examples are seen with the VM202 selected variants -- VM202(PY102-VP2)7 and VM202(PY102-VP5)16. In these cases binding of VM201 is largely unaffected, while binding of VM202 is greatly reduced.

3. CHARACTERIZATION OF V GENES USED BY MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL VIRUS VARIANTS

Table VI. HI titers - fine specificity of monoclonal antibodies to sequential PR8 virus variants

Virus	Monoclonal Antibodies					
	PY102	VM201	VM202	VM113	PY202	VM105[a]
A. PR8 plaque 2	11[b]	12	10	0	12	ND[c]
PY102-VP2[d]	0	12	9	1	ND	11
VM201-VP2	2	0	0	ND	ND	ND
VM202-VP2	2	0	0	ND	ND	ND
B. PR8 plaque 5	10	12	12	1	ND	12
PY102-VP5	1	12	9	2	ND	11
VM201-VP5	4	0	0	1	ND	10
VM202-VP5	3	0	0	2	ND	9

[a]RDE-treated ascites

[b]$-\log_2$ of highest dilution of ascites at which hemagglutination is inhibited

[c]Not determined

[d]Variant of PR8 virus plaque 2 that has been selected with PY102

Table VII. HI titers - fine specificity of monoclonal antibodies to sequential PR8 virus variants

Virus	Monoclonal Antibodies					
	PY102	VM201	VM202	VM113	PY202	VM105[a]
A. PR8 plaque 2	11[b]	12	10	0	12	ND[c]
VM201(PY102-VP2)1[d]	0	0	0	1	11	ND
VM202(PY102-VP2)7	0	9	0	2	ND	ND
B. PR8 plaque 5	10	12	12	1	ND	12
VM201(PY102-VP5)10	0	0	5	ND	12	ND
VM202(PY102-VP5)16	0	9	3	0	11	ND

[a]RDE-treated ascites

[b]$-\log_2$ of highest dilution of ascites at which hemagglutination is inhibited

[c]Not determined

[d]Variant of PR8 virus plaque 2 that was selected with PY102 and then further selected with VM201.

a. V_K GENES

It is well known that certain V_L genes are often required for the formation of particular immunoglobulin combining sites and consequently antigen specificities(Kabat et al., 1983). While approximately 8.6% of normal BALB/c K-bearing serum immunoglobulin utilizes the V_K21 group of light chains(Julius et al., 1981), Staudt and Gerhard(1983) and Moran et al.(manuscript in preparation) have found that 22% and 47%, respectively, of their anti-influenza monoclonal antibodies are V_K21^+. Furthermore, sequencing analysis of germ line V_K21 DNA segments suggests that each subgroup is defined by a single germ line segment(Heinrich et al., 1984). Consequently, we examined our monoclonal antibodies directed to sequential virus variants particularly for the presence of this light chain group.

We used a rabbit antiserum specific for V_K21 subgroups A, D, E and F, and to a lesser extent subgroup C, to establish a competition RIA. These results are shown in Figure 3. PC2880(V_K21 A) achieved 50% inhibition at 0.02 micrograms/ml, while with PC10916(V_K21 C), LPC1(V_K11) and PY203(lambda) this point occurred at 1.20, 1.50 and greater than 10 micrograms/ml, respectively. VM113(0.18 micrograms/ml), PY102(0.25 micrograms/ml) and VM201(0.40 micrograms/ml)

are quite similar both in terms of their 50% inhibition points and also the slope of the curves generated in the inhibition assay. They inhibit significantly better than PC10916 and therefore appear to use light chains of V_K21 subgroups A, D, E or F. VM202 does not appear to use a V_K21 light chain.

b. V_H GENES

Recently, Brodeur and Riblet(1984) estimated that the mouse IgH-V locus consists of approximately 100 V_H genes. These genes have been grouped into 7 V_H families on the basis of protein(Dildrop, 1984) and DNA sequence, and also Southern blot analysis of genomic DNA(Brodeur and Riblet, 1984; Brodeur et al., 1984). We have used the slot blotting technique to characterize the V_H families of our monoclonal antibodies.

The V_H families utilized by 3 of the 4 monoclonal antibodies specific for sequential virus variants have been determined(Figures 4-11). PY102 and VM202 hybridize most strongly with the 7183 probe(Figure 4) and show minimal hybridization with any of the other probes. VM201 hybridizes most strongly with the S107 probe(Figure 5) and minimally with the other probes. The V_H gene family of VM113 has not yet been determined by slot blot analysis. We know that this hybridoma is a low secretor of antibody and also a lower producer of mRNA compared to the other hybridomas tested here. Though some VM113 RNA

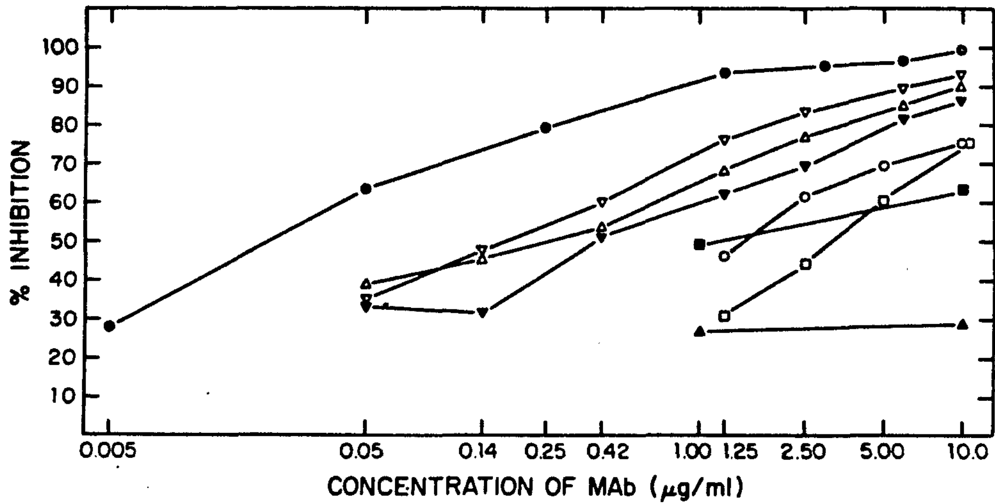

Figure 3. Inhibition of binding of ^{125}I-PC2880(V_K21A) to rabbit-anti-V_K21 A, D, E or F antibodies with various monoclonal antibodies at a range of concentrations. PC2880(V_K21A -●, PC10916(V_K21C) -○, LPC1(V_K11) -■, PY203(lambda) -▲, PY102 -△, VM113 - ▽, VM201 - ▼ and VM202 -□.

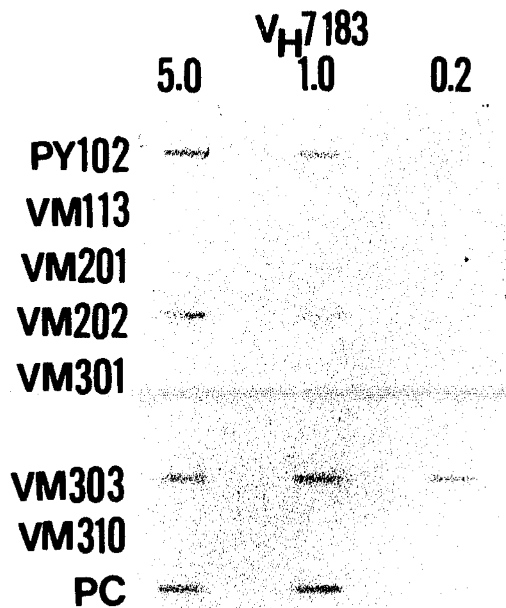

Figure 4. Hybridization of ^{32}P-V_H7183 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_H7183 positive control(PC) is MRL22-46.

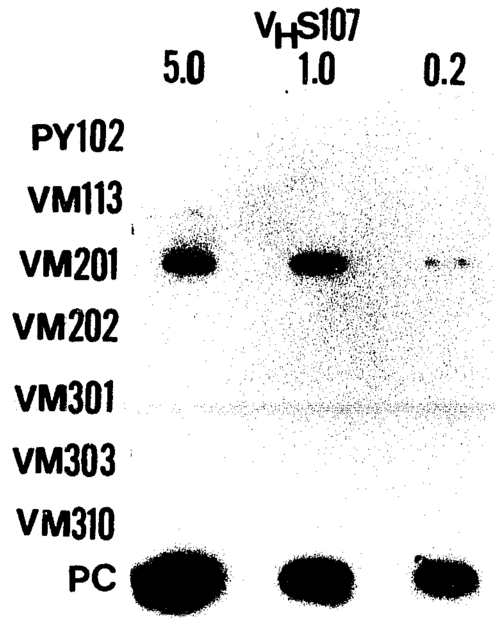

Figure 5. Hybridization of ^{32}P-V_HS107 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_HS107 positive control(PC) is HOPC8.

has been blotted as detected by a J_H probe(Figure 6), it appears that ultimate determination of its V_H family is a quantitative problem that will be resolved by blotting more RNA and using fresh probes of high specific activity. The possibility also exists that the particular V_H gene used by VM113 has a sequence which does not permit effective hybridization to any of the probes employed.

The V_H genes of VM301 and VM310 belong to the 36-60(Figure 7) and J558(Figure 8) families, respectively. VM303 hybridizes to both the 7183(Figure 4) and 441-4(Figure 9) probes. Because PY102 and VM202(both V_H 7183^+) do not cross hybridize with the 441-4 probe, the V_H gene of VM303 is clearly different from those of PY102 and VM202 and is more likely to be a member of the 441-4 family. None of the monoclonal antibodies examined utilize genes from the J606(Figure 10) or Q52(Figure 11) V_H gene families. The other monoclonal antibodies used in this study have been typed by Dr. Roy Riblet(Institute for Cancer Research, Philadelphia, Pa.).

4. IDIOTYPY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

Rabbit anti-idiotype reagents were prepared to PY102, VM113 and VM201. The anti-idiotype antibodies were eluted from affinity columns in which the antibody coupled to Sepharose was PY102, VM202, VM113 or VM201.

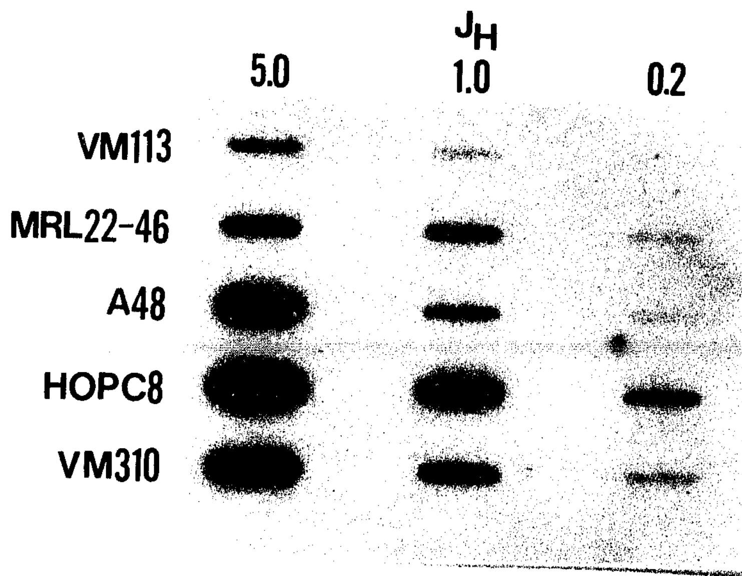

Figure 6. Hybridization of ^{32}P-J_H cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml.

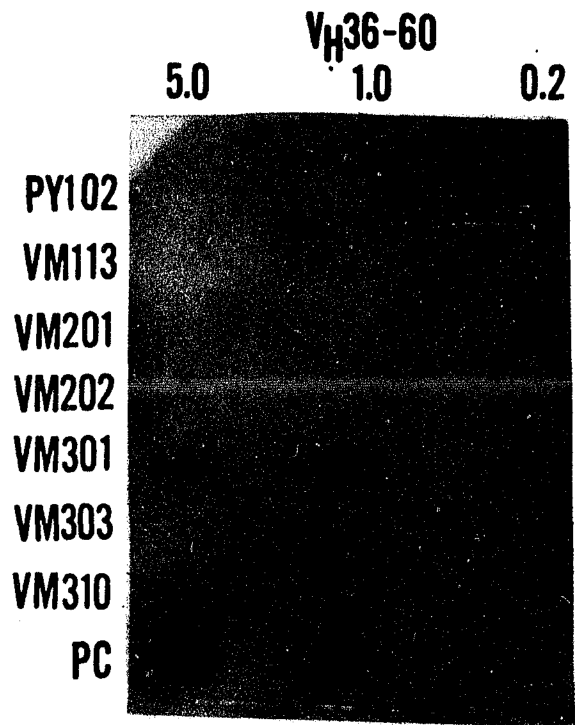

Figure 7. Hybridization of ^{32}P-V_H36-60 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_H36-60 positive control(PC) is 36-60.

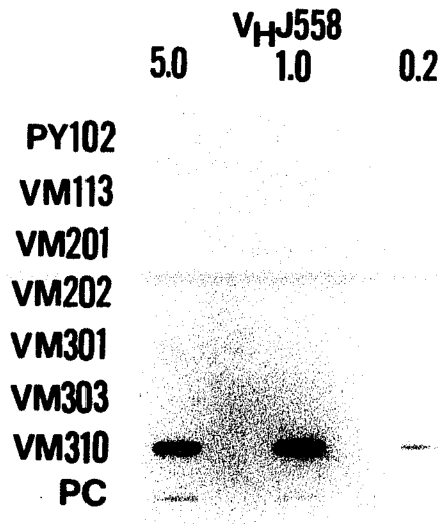

Figure 8. Hybridization of $^{32}P-V_HJ558$ cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_HJ558 positive control(PC) is J558.

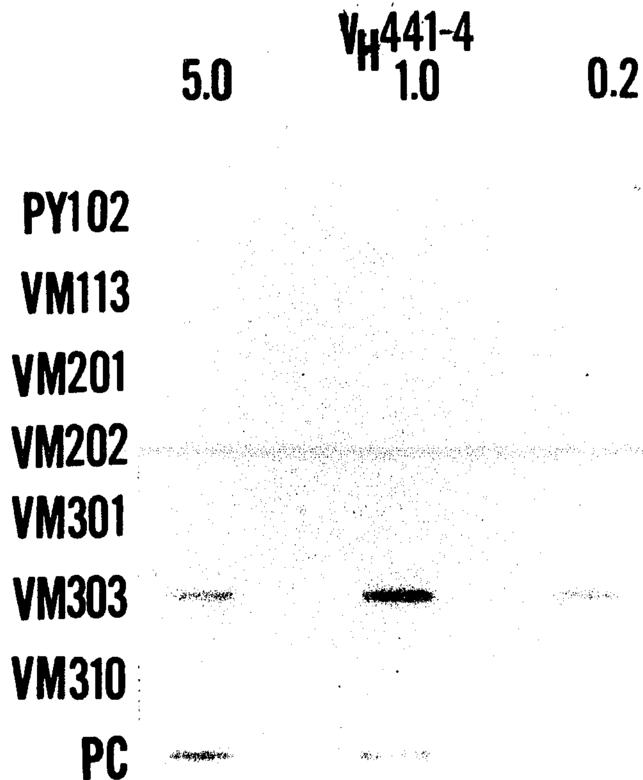

Figure 9. Hybridization of ^{32}P-V_H441-4 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_H441-4 positive control(PC) is A48.

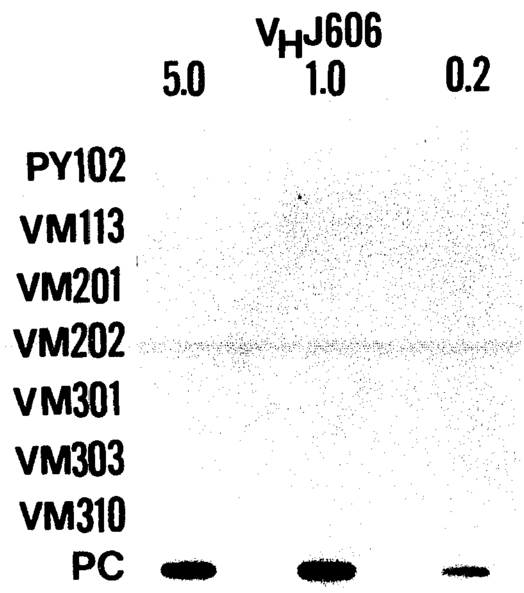

Figure 10. Hybridization of ^{32}P-V_HJ606 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_HJ606 positive control(PC) is J606.

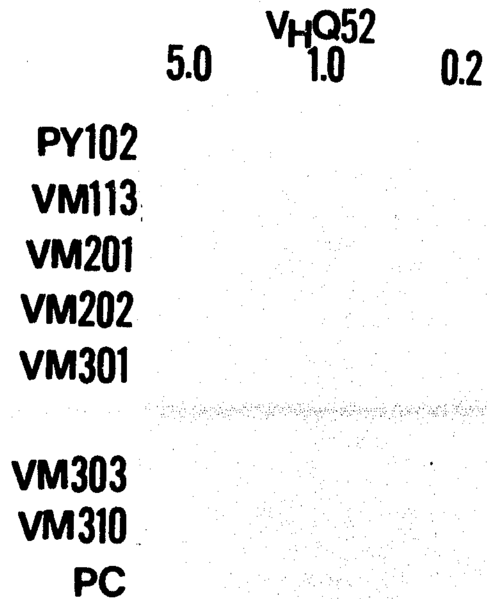

Figure 11. Hybridization of ^{32}P-V_HQ52 cDNA probe with RNA from various monoclonal antibodies. Guanidine thiocyanate-gradient purified RNA has been blotted at concentrations of 5, 1 and 0.2 micrograms/ml. The V_HQ52 positive control(PC) is MRL5-51.

Anti-isotype and anti-allotype antibodies were removed by passages over a Sepharose-BALB/c normal serum immunoglobulin column until there was no significant binding detectable.

In preliminary studies with the rabbit-anti-PY102 serum it was found that antibody eluted from a Sepharose-PY102 column bound to PY102, but that this binding could not be inhibited by antibodies other than PY102(data not shown). These data indicate that a major component of the rabbit-anti-PY102 antibody was directed to an individual idiotope, or that the affinity of anti-IdX antibodies for PY102 was significantly greater than their affinity for other antibodies.

Other studies with a BALB/c-anti-PY102 serum indicated a very slight cross reactivity between PY102 and VM202 in a sandwich assay(data not shown). Because of the limited quantity of this syngeneic antiserum, we attempted to enrich for the presumed anti-IdX activity in the more abundant rabbit-anti-PY102 serum by purification on a Sepharose-VM202 column. This purification was successful and therefore clearly indicated that PY102 and VM202 share an idiotype. Because these 2 monoclonal antibodies had been shown to use V_H genes from the 7183 family(Moran et al., manuscript in preparation), we also examined other influenza-specific monoclonal antibodies that had been typed to this family -- PT109,

XY101 and VM303.

Table VIII indicates that PY102, VM202, VM113, PT109 and XY101 show significant inhibitory ability and therefore share an idiotype. Variation in the amount of antibody needed to achieve 50% inhibition can probably be explained by differences in avidity with which the anti-idiotype antibodies bind to the different monoclonal antibodies. In addition, it is possible that different V_H germ line genes within the 12 member 7183 family(Brodeur and Riblet, 1984) are used by the different monoclonal antibodies. These explanations are not mutually exclusive. VM303 clearly shows no inhibitory activity. There is also no significant inhibitory activity among the remaining panel of monoclonal antibodies, particularly those that have been typed to the S107, J558 and 36-60 families.

The anti-idiotype antibodies obtained by immunization with VM113 were used in a sandwich assay(Table IX). The cross reactivity between PY102 and VM113 seen with the rabbit-anti-PY102 reagent(Table VIII) is not seen in this system -- only VM113 coated wells show significant binding. This indicates that VM113 bears an individual idiotype, or that the polyclonal anti-idiotype response in the rabbit immunized with VM113 was less cross reactive than that of the rabbit immunized with PY102.

Table VIII. Inhibition of binding of ^{125}I-VM202 to rabbit-anti-PY102 idiotype antibodies with various monoclonal antibodies

MAb	50% Inhibition(micrograms/ml)	V_H
PY102	1.6	7183
VM113	5.0	-
VM201	31.3	S107
VM202	1.6	7183
PT109	10.3	7183
XY101	11.9	7183
VM303	50.0	7183/441-4[a]
H36-5-3	50.0	S107
PY211	50.0	J558
VM310	50.0	J558
VM301	50.0	36-60
B109	50.0	N.D.

[a]RNA hybridizes with probes from both families

[b]Not determined

Table IX. Binding of rabbit-anti-VM113 idiotype antibodies to various monoclonal antibodies in a sandwich assay

Microtiter plate coated with MAb (20 micrograms/ml):	^{125}I-VM113 R-anti-VM113
PY102	429(38)[a]
VM113	3284(166)
VM201	475(49)
VM202	467(13)
VM310	439(5)
PY211	362(11)
VM301	445(38)
B109	359(18)
PBS	344(54)

[a] cpm=mean(SEM) of duplicates

Preliminary studies with the anti-idiotype antibodies directed to VM201 did not detect any cross reactivity among the monoclonal antibodies specific for sequential virus variants. Because this panel of monoclonal antibodies did not include any heavy chains from the S107 V_H family, we looked further at influenza binding and phosphorylcholine(PC) binding monoclonal antibodies that utilize this V_H family. The results of a sandwich assay are shown in Table X. The 3 influenza-specific monoclonal antibodies that utilize genes of the S107 V_H family -- VM201, H36-5-3 and H36-7-3 -- bind best to the rabbit-anti-VM201 reagent. The PC binding monoclonal antibodies vary widely in their binding to the anti-idiotype, though none bind as well as the influenza-specific, $S107^+$ monoclonal antibodies. Influenza-specific monoclonal antibodies from the 7183, 36-60 and J558 families do not show significant binding.

A competitive RIA was set up in which inhibition of binding of ^{125}I-H36-5-3 to the rabbit-anti-VM201 reagent was determined(Table XI). Only VM201 and H36-5-3 showed significant inhibition. The discrepant results of these 2 assay systems most likely reflect the greater sensitivity and lesser specificity of the sandwich assay relative to the less sensitive, more specific competitive RIA. That is, V_HS107^+ antibodies may have sufficient sequence homology to VM201 to be detected in the sandwich

Table X. Binding of rabbit-anti-VM201 idiotype antibodies to various monoclonal antibodies in a sandwich assay

Microtiter plate coated with MAb (10 micrograms/ml):	^{125}I-VM201 R-anti-VM201	V_H
PY102	472(13)[a]	7183
VM113	471(9)	ND[b]
VM201	4529(101)	S107
VM202	254(9)	7183
H36-5-3	1545(110)	S107
H36-7-3	1311(217)	S107
HPCG15	767(37)	S107
HPCG8	762(75)	S107
HPCG14	689(43)	S107
HPCG11	636(2)	S107
HPCG10	472(36)	S107
HPCG9	410(36)	S107
HPCG17	281(12)	S107
HPCM7	186(24)	S107
HPCM27	143(16)	S107
W3207	101(2)	S107

Table X(cont.).

Microtiter plate coated with MAb (10 micrograms/ml):	^{125}I-VM201 R-anti-VM201	V_H
VM310	592(246)	J558
PY211	189(27)	J558
VM301	307(36)	36-60
B109	324(9)	ND

[a]cpm=mean(SEM) of duplicates

[b]Not determined

Table XI. Inhibition of binding of ^{125}I-H36-5-3 to rabbit-anti-VM201 idiotype antibodies with various monoclonal antibodies

MAb	50% Inhibition(micrograms/ml)
PY102	>20
VM113	>20
VM201	0.78
VM202	>20
H36-5-3	0.10
H36-7-3	>20
HPCG15	>20
HPCG8	>20
HPCG14	>20
HPCG11	>20
HPCG10	>20
HPCG9	>20
HPCG17	>20
HPCM7	>20
HPCM27	>20
W3207	>20
VM310	>20
PY211	>20
VM301	>20
B109	>20

assay. However, in the more stringent competitive assay sequence differences with VM201 and/or H36-5-3 results in markedly reduced inhibitory activity. The use of different germ line genes within the S107 V_H family by these monoclonal antibodies would also be consistent with the above hypothesis.

Four monoclonal anti-idiotopes had previously been obtained to PY206 and PY211, both of which use genes of the J558 V_H family(Moran et al., manuscript in preparation). SN3-1A, SN3-9A and SP3-5A are monoclonal anti-idiotope antibodies specific for idiotopes on PY206, a monoclonal antibody directed to the H3 HA of X31 virus, while 63-4 is specific for an idiotope on PY211, a monoclonal antibody directed to the H1 HA of PR8 virus(see below). When the monoclonal antibodies specific for sequential virus variants were examined by these anti-idiotope reagents in both a sandwich and a competitive RIA, they were all found to be negative(Tables XII-XV).

Table XVI summarizes the V_H and V_L genes, specificity and idiotypy of monoclonal antibodies used in this study.

B. ANTI-IDIOTYPES AS IMMUNOGENS

1. CHARACTERIZATION OF MONOCLONAL ANTI-IDIOTOPE REAGENTS

A series of monoclonal anti-idiotope reagents was obtained by T. Moran(manuscript in preparation). SN3-1A,

Table XII. Binding of monoclonal anti-PY206 idiotope antibodies (SN3-1A, SN3-9A and SP3-5A) to various monoclonal antibodies in a sandwich assay

Microtiter plate coated with MAb (10 micrograms/ml)	^{125}I-PY206 SN3-1A	SN3-9A	SP3-5A
PY102	194(73)	401(57)	119(11)[a]
VM113	310(12)	692(165)	339(90)
VM201	80(1)	153(16)	-46(5)
VM202	187(29)	86(87)	298(25)
PY206	4467(178)	1695(317)	1412(23)
PY211	192(19)	433(30)	248(30)
VM301	209(36)	223(7)	75(14)
VM303	183(33)	83(39)	52(39)
VM310	226(146)	599(133)	145
BY104	202(3)	278(67)	148(28)
B109	111(26)	81(19)	124(25)

[a]cpm=mean(SEM) of duplicates

Table XIII. Binding of a monoclonal anti-PY211 idiotope antibody(63-4) to various monoclonal antibodies in a sandwich assay

Microtiter plate coated with MAb (20 micrograms/ml)	^{125}I-PY211 63-4
PY102	401(23)[a]
VM113	542(125)
VM201	324(57)
VM202	321(46)
PY206	446(24)
PY211	1635(138)
VM301	382(133)
VM303	271(47)
VM310	583(8)
BY104	134(17)
B109	294(39)

[a]cpm=mean(SEM) of duplicates

Table XIV. Inhibition of binding of ^{125}I-PY206 to monoclonal anti-PY206 idiotope antibodies with various monoclonal antibodies

Monoclonal Antibody	50% Inhibition(micrograms/ml)		
	SN3-1A[a]	SN3-9A	SP3-5A
PY102	>25	>25	>25
VM113	>25	>25	>25
VM201	>25	>25	>25
VM202	>25	>25	>25
PY206	0.3	0.3	<0.3
PY211	>25	>25	>25
VM301	>25	>25	>25
VM303	>25	>25	>25
VM310	>25	>25	>25
BY104	>25	>25	>25
B109	>25	>25	>25

[a] SN3-1A, SN3-9A and SP3-5A are monoclonal antibodies specific for idiotopes on PY206

Table XV. Inhibition of binding of ^{125}I-PY211 to a monoclonal anti-PY211 idiotope antibody with various monoclonal antibodies

Monoclonal Antibody	50% Inhibition(micrograms/ml) 63-4[a]
PY102	>25
VM113	>25
VM201	>25
VM202	>25
PY206	>25
PY211	<0.3
VM301	>25
VM303	>25
VM310	>25
BY104	>25
B109	>25

[a]Monoclonal antibody specific for a PY211 idiotope

Table XVI. Monoclonal antibodies used in the study of monoclonal antibodies specific for sequential PR8 virus variants

MAb	V_H	V_K21	PY102 IdX	VM201 IdX	Specificity
PY102	7183	ADEF	+	-	H1[a]
VM113	ND[b]	ADEF	+	-	H1
VM201	S107	ADEF	-	+	H1
VM202	7183	-	+	-	H1
H36-5-3	S107(V11)	C	ND	+	H1
H36-7-3	S107(V11)	C	ND	+	H1
HPCG15	S107(V1)	-	ND	-	PC[c]
HPCG8	S107(V1)	-	ND	-	PC
HPCG14	S107(V1)	-	ND	-	PC
HPCG11	S107(V1)	-	ND	-	PC
HPCG10	S107(V1)	-	ND	-	PC
HPCG9	S107(V1)	-	ND	-	PC
HPCG17	S107(V1)	-	ND	-	PC
HPCM7	S107(V1)	-	ND	-	PC
HPCM27	S107(V1)	-	ND	-	PC
W3207	S107(V1)	-	ND	-	PC

Table XVI(cont.).

MAb	V_H	$V_\kappa 21$	PY102 IdX	VM201 IdX	Specificity
PT109	7183	ADEF	+	-	H1(HA-2)[d]
XY101	7183	ADEF	+	-	H3
VM303	7183[e] 441-4	-	-	-	H1
PY206	J558	-	-	-	H3
VM310	J558	-	-	-	H1
PY211	J558	ADEF	-	-	H1
VM301	36-60	-	-	-	H1
B109	ND	ND	-	-	B[f]

[a]hemagglutinin subtype

[b]Not determined

[c]Phosphorylcholine

[d]Carboxy terminal cleavage product of hemagglutinin

[e]Hybridizes to both 7183 and 441-4 V_H probes

[f]Influenza B virus hemagglutinin

SN3-9A and SP3-5A are monoclonal anti-idiotopes specific for PY206, a monoclonal antibody directed to the X-31 hemagglutinin, and 63-4 is a monoclonal anti-idiotope specific for PY211, a monoclonal antibody directed to the PR8 hemagglutinin. Sharing of idiotypes among PR8 and X-31-specific monoclonal antibodies has been shown with the monoclonal anti-PY206 reagents in a sandwich assay(Moran et al., manuscript in preparation).

a. SERUM STUDIES

We decided to examine immune sera for the presence of these idiotopes for the following reasons:

i. Findings with monoclonal antibodies may not be representative in that exceedingly rare B cell clones can be immortalized.

ii. In anticipation of attempts to manipulate the influenza immune response in vivo with the monoclonal anti-idiotope reagents, it was essential to know if the idiotopes were represented in immune sera and to determine their particular specificities. It was also necessary to establish whether or not we had a sensitive detection system.

BALB/c mice were parenterally primed and then boosted 30 days later with 10 micrograms of purified PR8 or X-31 virus. Sera from the secondary PR8(10 days post-boost) and X-31(15 days post boost) immune responses were examined in an HI assay and the 4 anti-idiotope

competitive RIA systems(Figure 12). HI titers of these sera with their respective immunogen ranged from 7 wells(1:2560) to 9 wells(1:10,240)(data not shown).

In the competitive RIA the ability of immune sera to inhibit the binding of ^{125}I-labeled idiotope antibodies to plates coated with their respective anti-idiotope was measured. Inhibition was quantified by calculating the percent decrease in counts bound relative to BALB/c normal mouse serum at the same dilution. More than 100 individual BALB/c normal mouse sera have been shown to be non-inhibitory in any of the 4 systems(data not shown).

The SN3-9A and SP3-5A systems are most interesting in that the idiotopes they react with are present in both PR8 and X-31 immune sera. In the SN3-1A and 63-4 systems significant inhibition is more restricted to PR8 immune sera.

In order to compare these sera more quantitatively, serum idiotope titers were determined by establishing the dilution endpoint at which 40% inhibition of binding of labeled idiotopes to monoclonal anti-idiotopes occurred(Table XVII). The inhibitory activity of most sera was diluted out at a 1:42 dilution. There are, however, 2 individual sera that are quite exceptional -- 103 and 104. Serum 103 has titers of 1:960(SN3-1A system), 1:1920(SN3-9A), 1:1600(SP3-5A) and 1:10(63-4). If we use the concentration at which 50% inhibition is

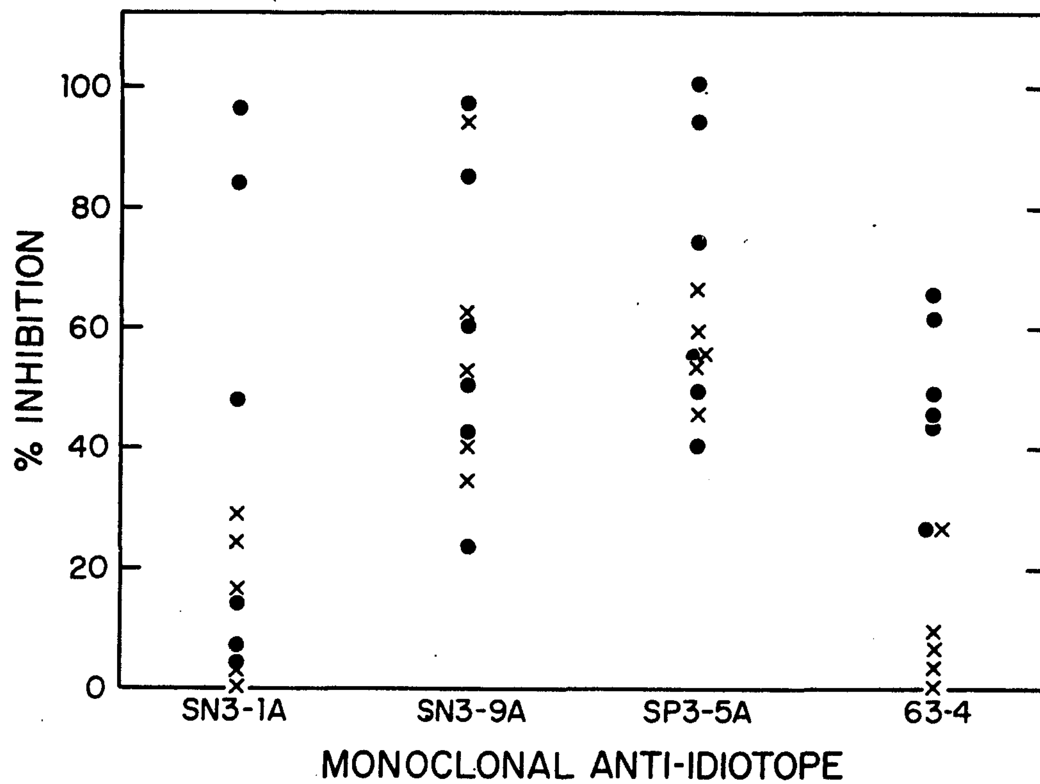

Figure 12. Inhibition of binding of ^{125}I-PY206 and ^{125}I-PY211 to their monoclonal anti-idiotopes -- SN3-1A, SN3-9A and SP3-5A, and 63-4, respectively -- with secondary PR8 and X-31 immune sera from individual BALB/c mice. PR8 immune sera - ●, X-31 immune sera - x.

achieved with purified PY206 in the SP3-5A system as a standard, the serum titer of 1:1600 roughly translates into 1.6 mg/ml. The titers of PY206 idiotopes in serum 103 exceed that of all other sera, except 104, by at least 38 fold. Serum 104 also has a very high titer of the SP3-5A defined idiotope, 1:320.

The inhibitory ability of sera from mice undergoing a primary response to X-31 virus(day 15 after priming) is shown in table XVIII. Though there is an occasional inhibitory serum -- 146 and 151(SN3-9A) and 146 and 147(63-4) -- these idiotopes are detected less frequently and at lower concentrations in a primary response relative to a secondary response to X-31 virus.

Having shown that the SN3-9A and SP3-5A defined PY206 idiotopes are borne by both PR8 and X-31 specific antibodies, we attempted to further define the range of different anti-influenza immune responses that might utilize these idiotopes. Groups of 8 BALB/c mice were each parenterally primed and then boosted 30 days later with 10 micrograms of purified B/Lee, X-31(H3N2), PR8(H1N1), A/Singapore(H2N2), A/Houston(H1N1) or A/Chicken(H5N1) viruses. Bleedings were done on days 3, 10, 20, 33, 37 and 44.

HI titers indicate that all groups made a classical primary and secondary humoral immune response to their respective immunogen with a general increase in titer

Table XVII. Serum idiotope titers of secondary PR8 and X-31 immune sera

Sera[a]	Monoclonal Anti-idiotopes SN3-1A	SN3-9A	SP3-5A	63-4
98[b]	<10[c]	<10	22[d]	<10
99	<10	30	30	10
101	<10	42	42	42
102	16	16	16	16
103	960	1920	1600	10
104	15	30	320	10
153	<10	10	15	<10
154	<10	15	10	<10
157-1	<10	10	10	<10
157-2	<10	<10	10	<10
158	<10	15	10	<10

[a]Sera 98-104(PR8 immune - 10 days post-boost); 153-158(X-31 immune sera - 15 days post-boost)

[b]Individual mouse designation

[c]No inhibition at a 10 fold dilution of serum

[d]40% inhibition at a 22 fold serum dilution

Table XVIII. Inhibition of binding of ^{125}I-PY206 or ^{125}I-PY211 to monoclonal anti-idiotopes by primary X-31 immune sera

Sera[a]	Monoclonal anti-idiotopes SN3-1A	SN3-9A	SP3-5A	63-4
37[b]	-10[c]	18	-33	17
38	-5	17	-18	4
145	-3	-4	-12	7
146	7	33	14	27
147	17	20	17	25
149	3	13	-7	6
150	10	8	-9	1
151	11	26	-4	2
152	1	23	-11	16
PC[d]	83	91	77	95

[a]Day 15 of primary immune response

[b]Individual mouse designation

[c]Percent inhibition

[d]PY206(SN3-1A, SN3-9A and SP3-5A systems) or PY211(63-4 system) hybridoma supernatants

after the boost(Figure 13). In terms of idiotope expression no significant inhibition was seen in the SN3-1A, SN3-9A and 63-4 systems in this experiment. The inhibitory activity in the SP3-5A system is shown in Figure 14. The most important point is that this idiotope is utilized in all 6 antiviral responses, including the response to B/Lee virus. The general pattern is that of an increase in inhibitory activity until a peak is reached at day 37 and then a decrease in inhibitory activity occurs. In the case of A/Singapore, A/Houston and A/Chicken viruses this final decrease is precipitous, while the HI titers remain at a plateau level. The initial general increase in inhibitory activity roughly correlates with the rise in HI activity, suggesting that the idiotope is borne by virus binding antibody.

Further definition of the nature of the serum idiotopes was important. Firstly, it was important to rigorously establish that these idiotopes do reside primarily on virus binding antibody and do not represent parallel sets of antibodies of diverse specificity coincidentally elicited following virus immunization(Liu et al., 1981).

Serum 103(secondary PR8 immune serum) was selected for adsorption studies because ot its extremely high titer(Table XVII). The ability of adsorbed and unadsorbed sera to bind to PR8 virus is shown in Figure

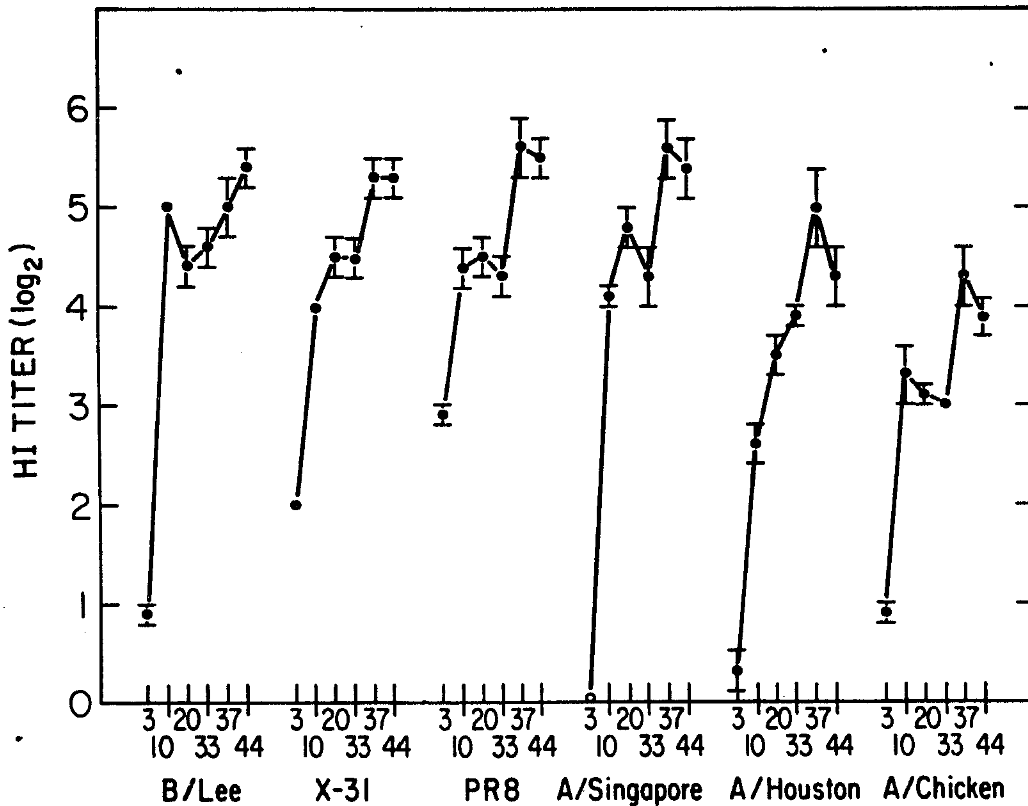

Figure 13. Hemagglutination inhibition titers of sera from BALB/c mice immunized with B/Lee, X-31(H3N2), PR8(H1N1), A/Singapore(H2N2), A/Houston(H1N1) and A/Chicken(H5N1) viruses. Mice were primed and then boosted on day 30 with 10 micrograms of gradient purified virus. Bleedings were done on days 3, 10, 20, 33, 37 and 40. The mean titer of 8 individual sera and the standard error of the mean(SEM) is presented.

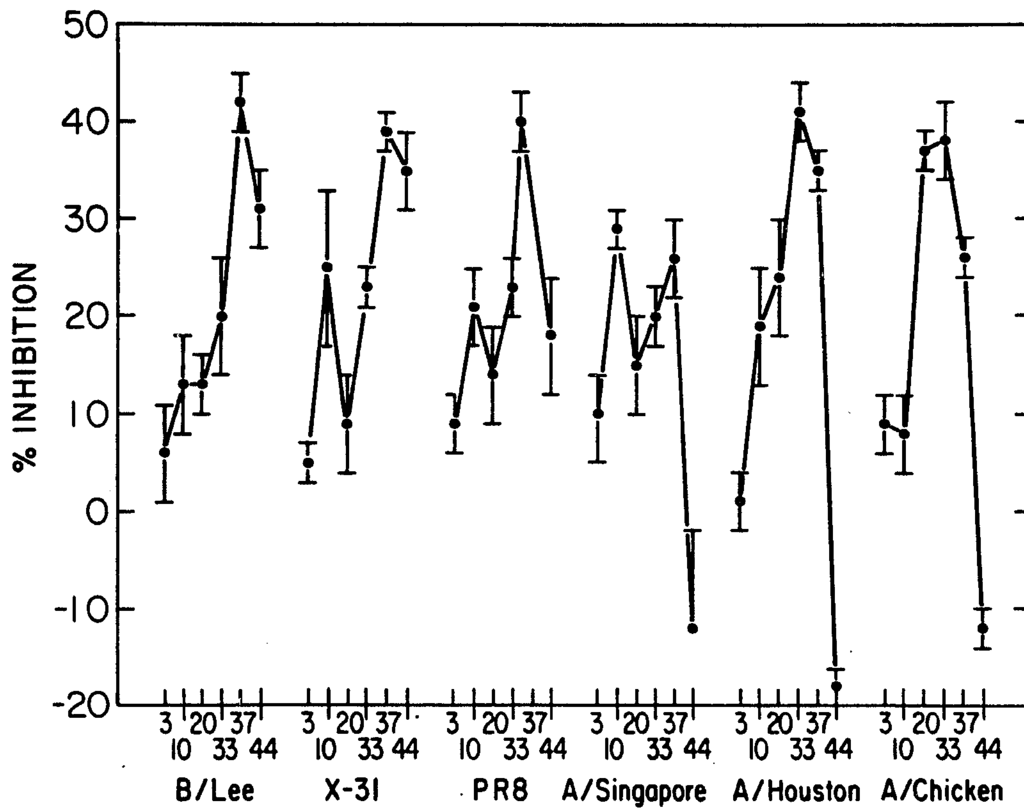

Figure 14. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope antibody(SP3-5A) with sera from BALB/c mice immunized with various viruses. Mice were primed and boosted 30 days later with 10 micrograms of gradient purified virus. Bleedings were done on days 3, 10, 20, 33, 37 and 40. The mean percent inhibition with standard error(SEM) is shown.

15. At all dilutions there has been significant removal of PR8 binding antibody. Where the curve is essentially linear(1:2560 and 1:5120), there has been removal of approximately 70% of the counts bound as a result of adsorption.

The effect of removal of PR8 binding antibody on the SP3-5A defined idiotope is shown in Figure 16. At the 2 highest dilutions, where the curves are most linear, there has been significant removal of the inhibitory activity of this serum after adsorption on virus. Inhibitory activity was not removed at the 2 lowest dilutions, eventhough virus binding activity was clearly removed. However, the absence of removal of inhibitory activity at the 2 lowest dilutions may not be so surprising because neither the curve of virus binding or inhibitory activity has a slope of 1. That is, in neither case is a change in the abscissa accompanied by an exactly equivalent change in the ordinate. We conclude that a significant proportion of the serum antibody bearing the SP3-5A defined idiotope is also virus specific.

Because only antibodies to the surface hemagglutinin and neuraminidase have significant protective ability, it was important to further define the specificity of these idiotope bearing antibodies. This was accomplished by adsorption on X-31 virus, a reassortant virus identical

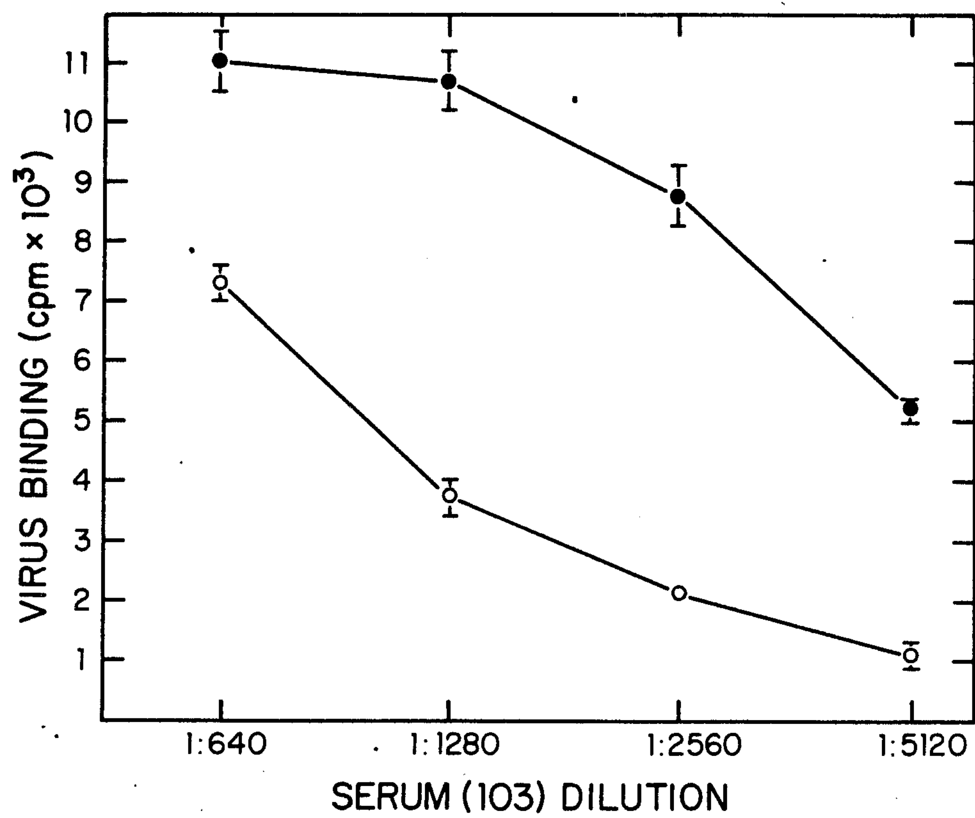

Figure 15. Binding of various dilutions of adsorbed and unadsorbed serum 103 to plates coated with PR8 virus. Adsorbed on PR8 virus coated plates - o; Unadsorbed - •. Values presented are mean(of duplicates)$\pm$SEM.

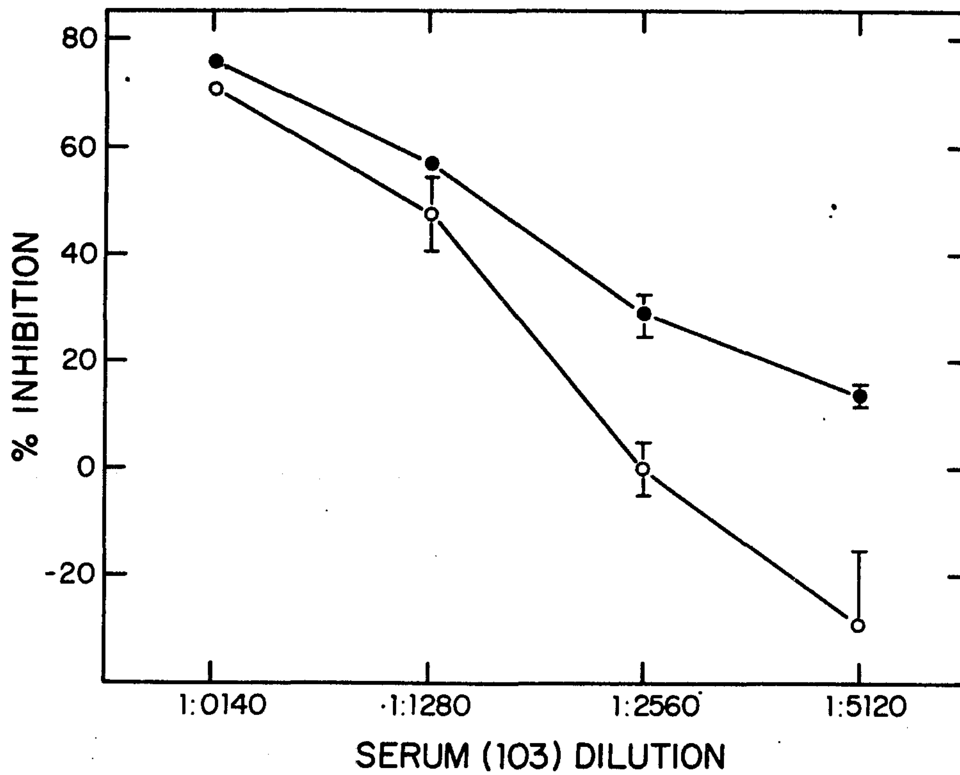

Figure 16. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope(SP3-5A) with various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on plates coated with PR8 virus - o; Unadsorbed - ●. Values presented are mean(of duplicates)±SEM.

to PR8 virus except for its surface glycoproteins(H3N2). Removal of antibody that binds to both PR8 and X31 and its effect on the idiotope would allow us to discriminate HA and/or NA-specific idiotope bearing antibody from idiotope bearing antibody that is specific for internal viral proteins.

The efficacy of the X-31 adsorption is shown in Figure 17. Again, at all dilutions there has been significant removal of X-31 virus binding antibody. In the most linear portion of the curve(1:640 and 1:1280), there has been removal of 60-70% of the counts bound.

The effect of this virus adsorption on the SP3-5A defined idiotope is shown in Figure 18. At no dilutions has removal of X-31 binding antibody resulted in removal of the idiotope. At 2 dilutions(1:640 and 1:2560) the X-31 adsorbed sera actually has more inhibitory activity than the unadsorbed sera, a phenomenon that was reproducible in several experiments. Consequently, the majority of antibodies bearing the SP3-5A defined idiotope are HA and/or NA specific.

Similar studies were also done with the SN3-9A anti-PY206 idiotope. In Figure 19 we see again that removal of PR8 virus binding antibody is associated with removal of idiotope bearing antibodies at the 3 highest dilutions(1:1280, 1:2560 and 1:5120). Adsorption on X-31 coated plates does not deplete the idiotope(Figure 20).

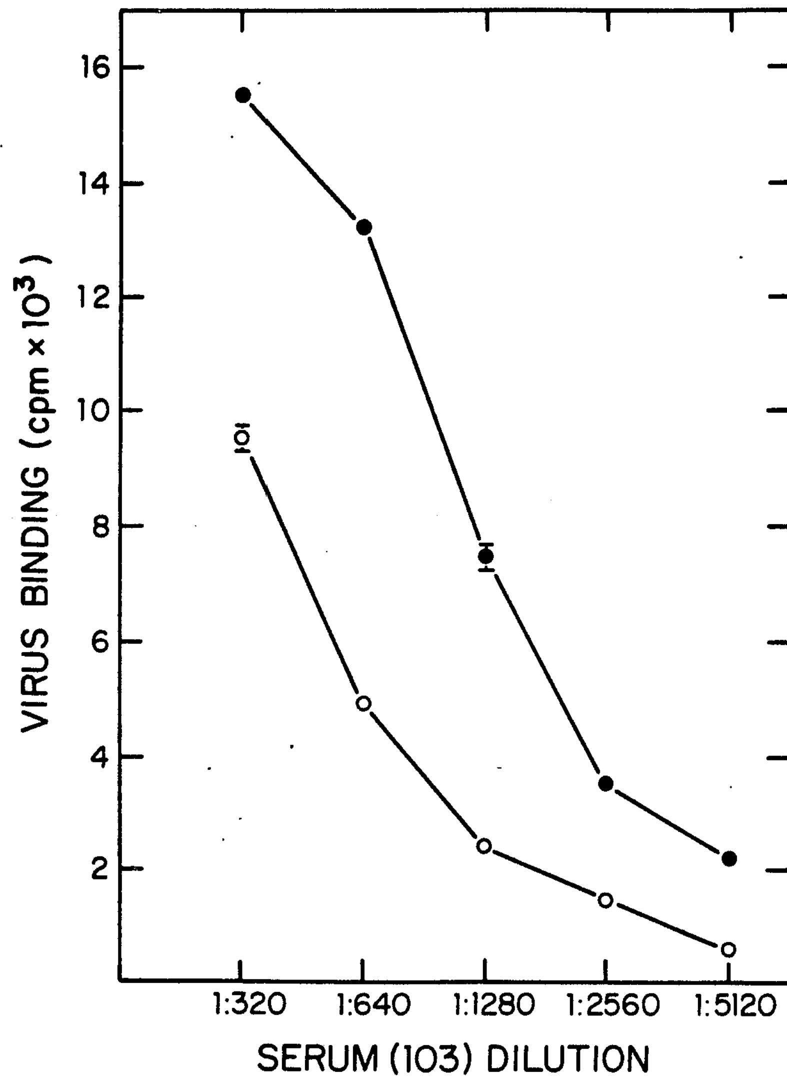

Figure 17. Binding of various dilutions of adsorbed and unadsorbed serum 103 to plates coated with X-31 virus. Adsorbed on plates coated with X-31 virus - o; Unadsorbed - ●; Values presented are means(of duplicates)±SEM.

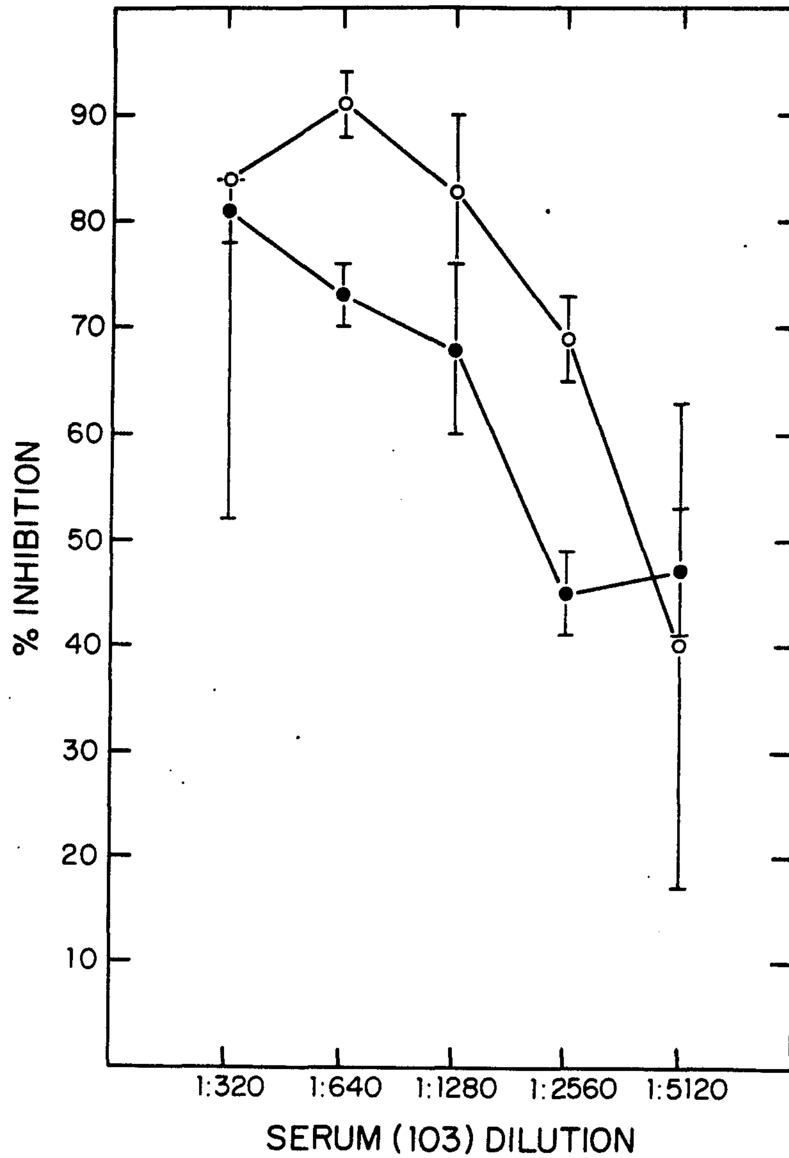

Figure 18. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope(SP3-5A) with various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on plates coated with X-31 virus - o; Unadsorbed - •. Values presented are mean(of duplicates)$\pm$SEM.

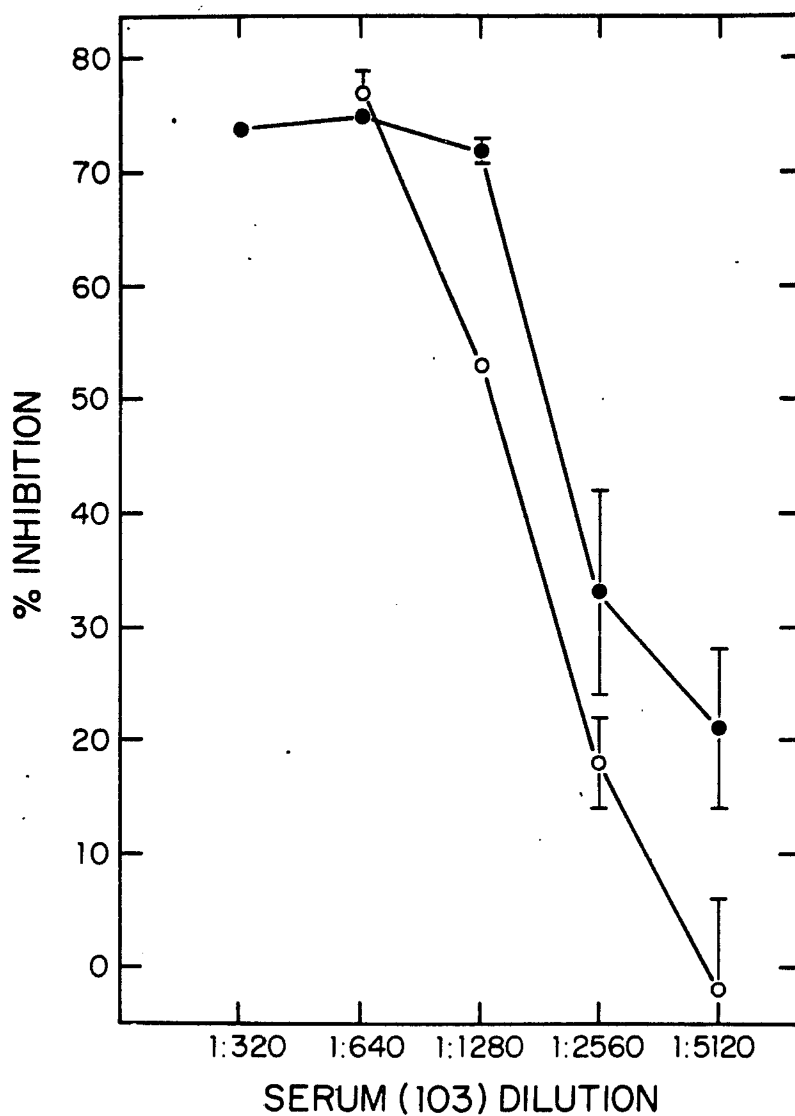

Figure 19. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope(SN3-9A) with various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on plates coated with PR8 virus - o; Unadsorbed - ●. Values presented are mean(of duplicates)$\pm$SEM.

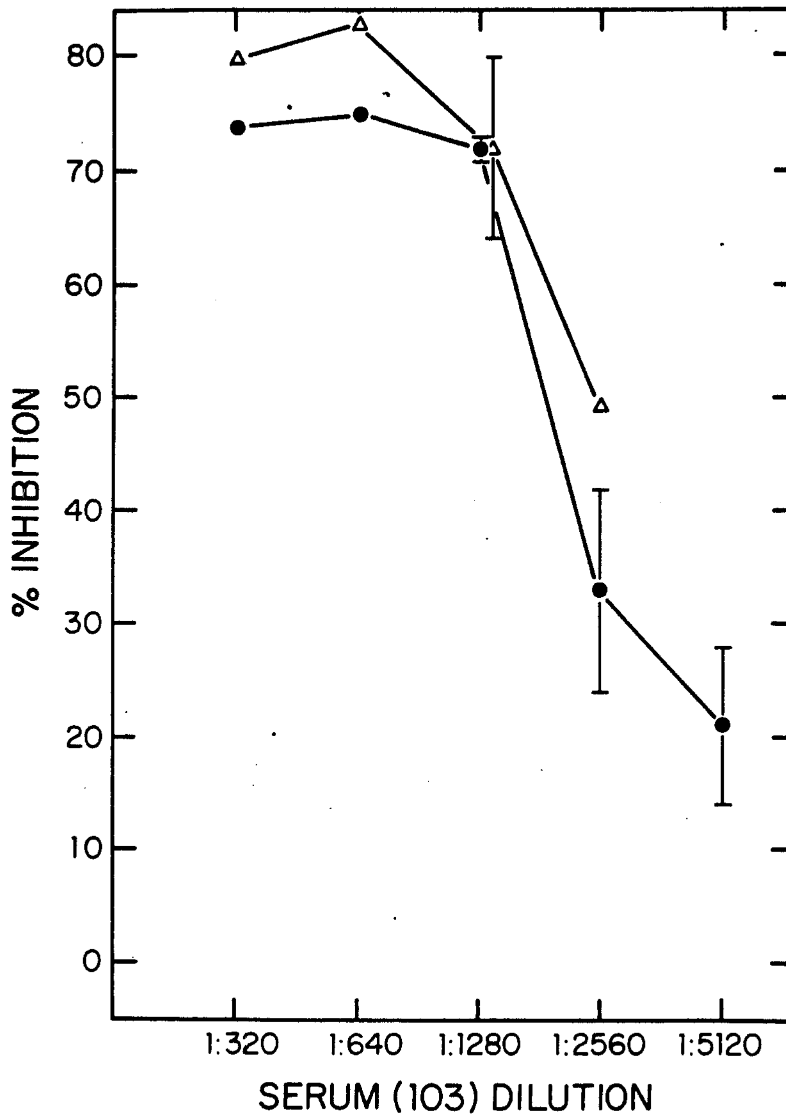

Figure 20. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope(SN3-9A) with various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on plates coated with X-31 virus - ▲; Unadsorbed - ●. Values presented are mean(of duplicates)±SEM.

Therefore, a significant proportion of antibodies bearing the SN3-9A idiotope are HA and/or NA specific.

Adsorption studies were also done in the SN3-1A system. These studies indicated that removal of virus binding antibodies resulted in removal of idiotope bearing antibodies at occasional dilutions, though there was no discernable dose dependence and it was not reproducible in several experiments(data not shown). Consequently, we are at present unable to conclude that antibodies bearing the SN3-1A defined idiotope are also virus binding antibodies. The 63-4 defined idiotope was also not examined because the lack of a high titer serum precluded use of serum dilutions at which efficient adsorption of antibody is observed..

The above studies allowed conclusions concerning the proportion of idiotope bearing antibodies that are virus binding. It did not tell us the proportion of virus binding antibodies that express these particular idiotopes. That is, do these idiotopes comprise a major or minor component of the total anti-viral response?

This question was answered in the SP3-5A system by adsorption on microtiter plates coated with SP3-5A. Removal of idiotope bearing antibody by this technique is clearly shown in Figure 21. Significant removal of idiotope occurs at all dilutions except 1:2560.

The effect of removal of the idiotope on the

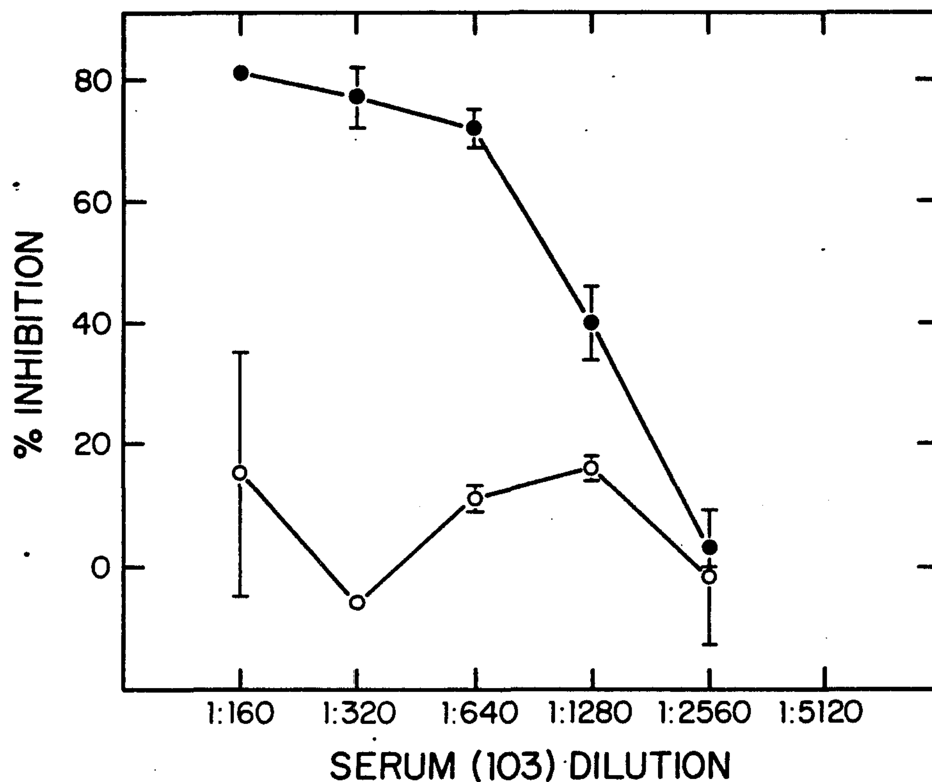

Figure 21. Inhibition of binding of ^{125}I-PY206 to a monoclonal anti-idiotope(SP3-5A) with various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on plates coated with SP3-5A - o; Unadsorbed - ●. Values presented are mean(of duplicates)$\pm$SEM.

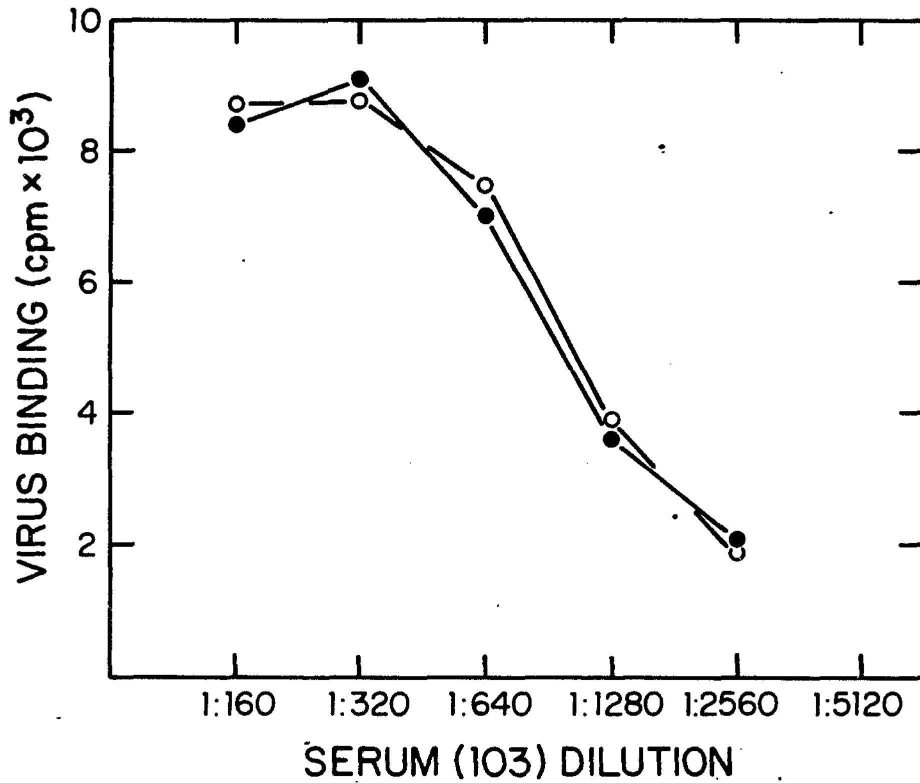

Figure 22. Binding of various dilutions of adsorbed and unadsorbed serum 103 to plates coated with PR8 virus. Adsorbed on SP3-5A coated plates - o; Unadsorbed - ●. Values presented are mean(of duplicates)±SEM.

influenza-specific antibody was examined in terms of both virus binding antibody(RIA) and HI antibody(Figures 22 and 23, respectively). The adsorption clearly had no effect on virus binding antibody. However, we do not consider the effect on total virus binding antibody the most sensitive and meaningful indicator in that the population of antibodies we are most interested in -- neutralizing antibody -- is diluted by antibodies to the internal viral proteins. The effect on HI antibody is really the more definitive indicator. However, this also was unaffected by idiotope removal(Figure 23). Therefore, the SP3-5A defined idiotope represents a minor component of the antibody response to influenza.

b. HUMAN MONOCLONAL ANTI-INFLUENZA ANTIBODIES

A panel of 27 EBV-transformed human B cell clones specific for PR8 virus was generated by Dr. Nolan Sigal(Merck, Sharp and Dohme, Rahway, N.J.). The viral protein specificities of these antibodies has not been defined. Supernatants were tested in the 4 monoclonal anti-idiotope systems(Table XIX). Because these EBV-transformed B cells are all IgM producers, it is important that a human monoclonal antibody be clearly negative in at least one system in order to rule out the possibility that its inhibition is solely a reflection of its isotype. Consequently, significant inhibition has been conservatively defined as at least 35% inhibition

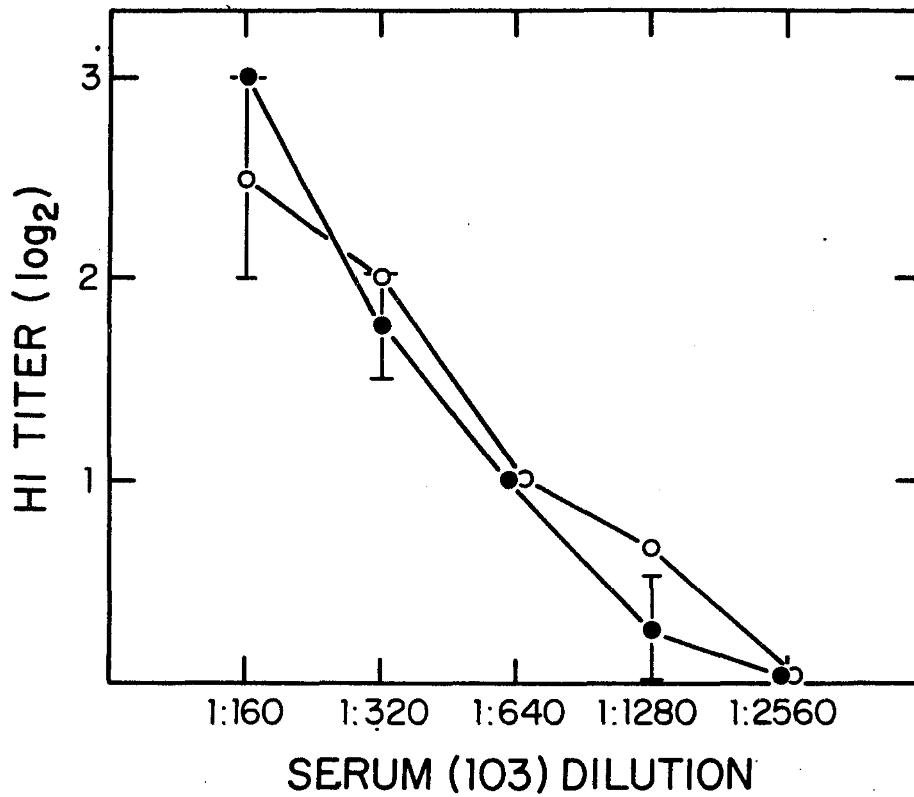

Figure 23. Hemagglutination inhibition titers of various dilutions of adsorbed and unadsorbed serum 103. Adsorbed on SP3-5A coated plates - o; Unadsorbed - ●. Values presented are mean(of duplicates)±SEM.

Table XIX. Inhibition of binding of ^{125}I-PY206 or PY211 to monoclonal anti-idiotopes with human monoclonal anti-influenza antibodies

	Monoclonal Anti-idiotopes			
HMAb #	SN3-1A	SN3-9A	SP3-5A	63-4
1	5[a]	-15	2	ND
2	-1	-21	-28	-24
3	-7	-25	-30	3
4	-5	-28	8	7
6	24	24	45	13
7	19	9	17	12
8	25	-46	7	-9
9	13	-47	0	4
10	15	-9	38	13
11	30	ND	ND	ND
12	33	9	46	20
13	23	-2	59	12
14	33	-4	46	13
15	22	10	32	16
16	14	-38	47	22
17	28	16	35	13
18	36	15	46	15
19	37	15	59	12
20	33	0	45	22

Table XIX(cont.).

HMAb #	SN3-1A	SN3-9A	SP3-5A	63-4
	Monoclonal Anti-idiotopes			
21	23	-10	49	26
22	14	6	-4	33
23	16	-5	20	-4
24	18	22	31	40
25	31	23	54	ND
26	39	32	64	ND
27	7	-21	31	34
28	4	-4	44	38
PY206[b]	90	86	87	ND
PY211[b]	ND	ND	ND	88

[a]Percent inhibition

[b]Positive controls - BALB/c anti-influenza hybridoma supernatants

that is also at least double the percent inhibition seen in any of the other systems. Therefore, the notable positive monoclonal antibodies are 10, 13 and 16 in the SP3-5A system.

2. ANTI-IDIOTOPE IMMUNIZATIONS

Our interest in eliciting an influenza specific response by immunization with anti-idiotype has precedent in 5 viral systems -- hepatitis B virus(Kennedy and Dreesman, 1984), reovirus(Sharpe et al., 1984), rabies virus(Reagan et al., 1983), Sendai virus (Ertl and Finberg, 1984) and Venezuelan equine encephalomyelitis virus(Roehring et al., 1984). The hepatitis system has been a useful model for our initial attempt.

The experimental protocol was as follows. BALB/c mice received 5, 1 and 0.1 micrograms of alum precipitated monoclonal anti-idiotopes(SN3-1A, SN3-9A, 63-4 and IDA-10) intraperitoneally. IDA-10 is a monoclonal anti-idiotope specific for A48, a bacterial levan binding monoclonal antibody. They then received intraperitoneally 10 micrograms of X-31 or PR8 virus either 7 or 14 days after anti-idiotope priming. All mice were then bled on days 7, 14 and 21 after their respective virus immunization to determine whether anti-idiotype immunization primed for subsequent antiviral responses..

Results of the HI assay are presented in Tables XX-

Table XX. HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with X-31 virus

MAId 5 micrograms/mouse	Days post-virus immunization 7	 14	 21
A.[a]SN3-1A	4.0(.3)[b]	4.0(.3)	4.6(.2)
SN3-9A	4.2(.2)	4.2(.2)	4.0(0)
63-4	4.0(0)	4.0(0)	4.2(.2)
IDA-10[c]	4.0(0)	3.8(.3)	4.0(0)
B.[d]SN3-1A	3.6(.4)	3.4(.2)	3.8(.4)
SN3-9A	3.2(.2)	3.2(.2)	3.6(.2)
63-4	4.0(.3)	4.3(.3)	3.8(.2)
IDA-10	3.4(.2)	3.3(.3)	3.2(.2)

[a]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 14 days after anti-idiotope immunization

Table XXI . HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with X-31 virus

MAId 1 microgram/mouse	Days post-virus immunization 7	14	21
A.[a]SN3-1A	4.0(0)[b]	3.8(.3)	4.5(.3)
SN3-9A	3.8(.2)	4.2(.4)	4.0(0)
63-4	4.0(0)	4.0(.4)	4.0(0)
IDA-10[c]	3.8(.5)	3.8(.2)	4.4(.2)
B.[d]SN3-1A	3.6(.2)	3.0(0)	3.2(.2)
SN3-9A	3.4(.4)	3.0(0)	3.0(0)
63-4	4.0(0)	3.4(.2)	3.4(.2)
IDA-10	3.4(.2)	3.4(.2)	3.8(.2)

[a]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 14 days after anti-idiotope immunization

Table XXII . HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with X-31 virus

MAId 0.1 micrograms/mouse	Days post-virus immunization 7	 14	 21
A.[a]SN3-1A	3.8(.2)[b]	3.6(.2)	4.4(.2)
SN3-9A	3.8(.2)	4.0(0)	4.2(.2)
63-4	4.0(0)	3.4(.2)	4.4(.2)
IDA-10[c]	4.0(.3)	3.8(.2)	4.2(.2)
B.[d]SN3-1A	3.4(.2)	2.8(.2)	3.0(.3)
SN3-9A	4.0(0)	3.6(.2)	3.6(.4)
63-4	4.0(0)	3.4(.2)	3.4(.2)
IDA-10	4.2(.2)	3.6(.2)	3.8(.2)

[a]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified X-31 virus/mouse) 14 days after anti-idiotope immunization

Table XXIII. HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with PR8 virus

MAId 5 micrograms/mouse	Days post-virus immunization 7	 14	 21
A.[a] SN3-1A	6.6(.2)[b]	6.8(.4)	8.6(.2)
SN3-9A	6.8(.4)	6.8(.5)	7.4(.2)
63-4	6.4(.2)	6.0(.3)	6.8(.4)
IDA-10[c]	6.0(0)	5.6(.4)	6.0(0)
B.[d] SN3-1A	6.0(0)	5.8(.2)	6.6(.4)
SN3-9A	6.0(.3)	6.2(.2)	6.0(0)
63-4	6.4(.2)	6.0(.2)	6.4(.2)
IDA-10	4.8(.6)	5.0(.4)	5.6(.7)

[a]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 14 days after anti-idiotope immunization

Table XXIV. HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with PR8 virus

MAId 1 microgram/mouse	Days post-virus immunization 7	 14	 21
A.[a]SN3-1A	6.4(.2)[b]	6.0(0)	7.2(.2)
SN3-9A	6.4(.2)	6.6(.2)	6.4(.2)
63-4	6.0(.3)	5.8(.2)	6.8(.4)
IDA-10[c]	5.6(.2)	6.0(0)	6.8(.4)
B.[d] SN3-1A	5.8(.4)	5.6(.2)	5.6(.4)
SN3-9A	6.6(.2)	6.0(.3)	6.4(.2)
63-4	6.4(.2)	5.6(.4)	5.8(.2)
IDA-10	6.0(.3)	5.6(.4)	5.4(.2)

[a]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 14 days after anti-idiotope immunization

Table XXV. HI titers of sera from BALB/c mice that had been primed with various monoclonal anti-idiotopes and then boosted with PR8 virus

MAId 0.1 micrograms/mouse	Days post-virus immunization 7	 14	 21
A.[a]SN3-1A	5.2(.5)[b]	5.6(.2)	6.8(.2)
SN3-9A	6.2(.2)	6.6(.2)	7.4(.2)
63-4	6.6(.4)	6.4(.2)	6.6(.4)
IDA-10[c]	6.0(.3)	4.8(.3)	5.8(.6)
B.[d]SN3-1A	6.2(.2)	5.8(.2)	6.6(.2)
SN3-9A	6.4(.2)	6.2(.2)	6.6(.2)
63-4	6.8(.4)	6.2(.2)	5.8(.4)
IDA-10	5.6(.2)	6.4(.2)	6.4(.2)

[a]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 7 days after anti-idiotope immunization

[b]$-\log_2$(SEM) of highest dilution at which hemagglutination is inhibited(5 mice/group)

[c]Monoclonal anti-idiotope directed to A48, a bacterial levan binding monoclonal antibody

[d]Virus immunization(10 micrograms gradient purified PR8 virus/mouse) 14 days after anti-idiotope immunization

XXV. No differences were seen in HI titers after immunization with SN3-1A, SN3-9A and 63-4 relative to the control monoclonal anti-idiotope(IDA-10). The apparent slightly increased titer in SN3-1A immunized mice on day 21 after PR8 immunization relative to IDA-10 immunized mice(Table XXIII, panel A) was not evident in another HI assay or by neutralization assay.

C. ISOTOPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT.

The xid defect, defined in the CBA/N strain of mice, interferes with the development of a B cell subset which displays the Lyb5 surface antigen(Scher, 1982). This is reflected in a variety of abnormalities, most notably the inability to respond to certain thymus independent antigens that have been designated type 2(Scher et al., 1975). C_3H/HeJ mice, which possess a mutant gene(Lps^d) responsible for their ability to respond to endotoxin(Watson and Riblet, 1975), have provided the genetic background for a congenic strain bearing the xid defect -- C_3J.xid(Bona et al., 1980). Interestingly, this strain bears all the defects of the parental CBA/N and C_3H/HeJ strains in an enhanced form and also additional defects. The effect of the xid defect on the humoral response to PR8 virus has been examined here.

1. PR8 VIRUS SPECIFIC ANTIBODY RESPONSE AFTER INTRAPERITONEAL IMMUNIZATION

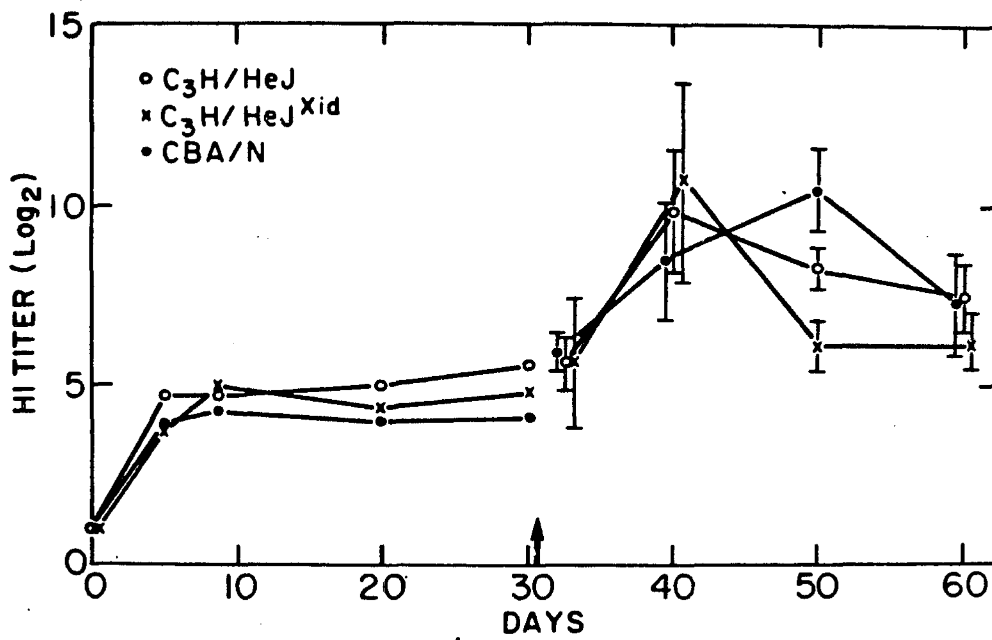

Figure 24. Hemagglutination inhibition titers(error bars are SEM) of individual serum specimens; 5 mice from each strain were immunized by intraperitoneal injection of viral protein on days 0 and 30. The mice underwent orbital bleeding at various times over a 60 day period. Arrow indicates secondary immunization on day 30; C_3H/HeJ(o), C_3H/HeJ$^{\underline{xid}}$ = C_3J.*xid*(x), CBA/N(•).

Mice from the CBA/N, C_3H/HeJ, and C_3J.*xid* strains received an intraperitoneal injection of 10 micrograms of purified PR8 virus on day 0 and were boosted on day 30 with the same dose. Serum samples were obtained by orbital bleeding on days 0, 5, 10, 20, 30(primary response), 33, 40, 50 and 60(secondary response).

The kinetics of HI antibody production during primary and secondary responses in the different strains are shown in Figure 24. No differences in the magnitude of response were observed.

Sera from five mice were pooled and employed in duplicate in a solid-phase isotype specific radioimmunoassay for a determination of the isotypes of anti-PR8 virus antibodies(Figure 25). The most significant differences among the strains were in production of the IgG_1 and IgG_3 subclasses of PR8 virus-specific antibodies. The IgG_1 subclass was detectable only in the C_3H/HeJ mice. The IgG_3 production of this strain also exceeded that of the CBA/N and C_3J.*xid* strains throughout both the primary and secondary responses. The CBA/N secondary IgG_3 response was somewhat greater than that of the C_3J.*xid*. The IgM, IgG_{2b} and IgG_{2a} responses were very similar in terms of kinetics and relative magnitudes in both the primary and secondary response in the different strains. One exception occurred on day 20 of the primary response,

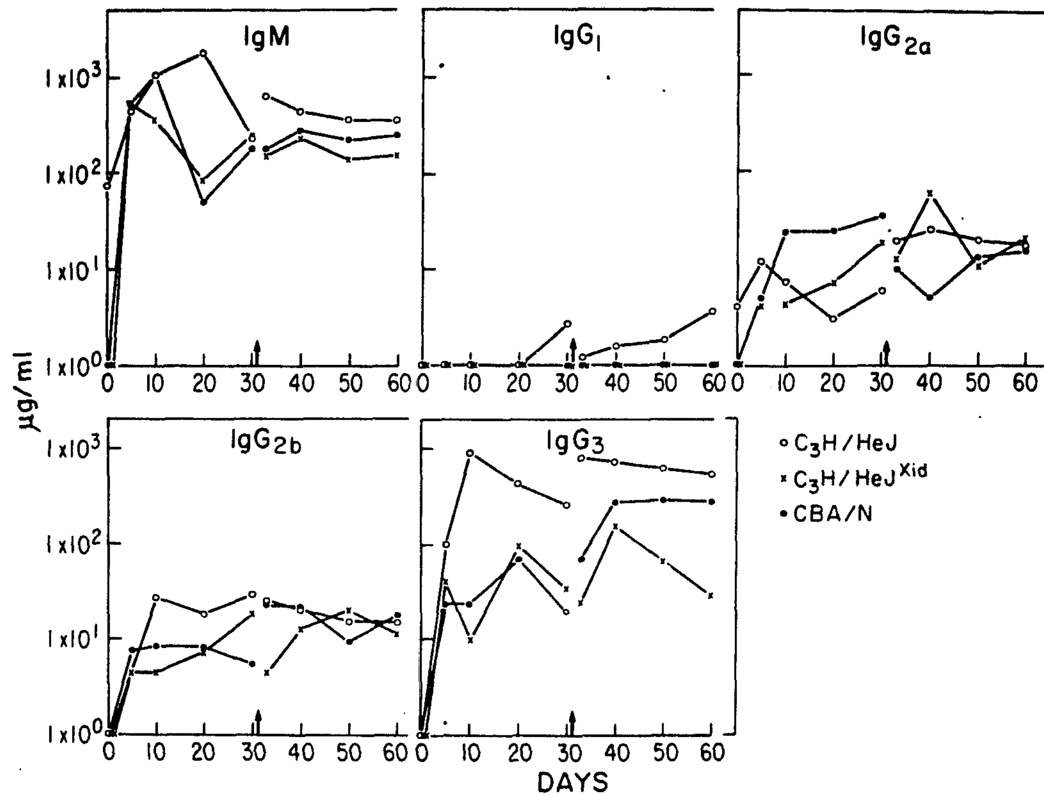

Figure 25. Isotype profiles of PR8 virus-specific antibodies following primary and secondary parenteral immunization in C_3H/HeJ(o), C_3H/HeJ$^{\underline{xid}}$ = C_3J.*xid*(x) and CBA/N(●) mice. Serum specimens were employed in a solid phase radioimmunoassay that utilized PR8 hemagglutinin-specific monoclonal antibodies of known isotype to develop standard curves and also ^3H-labeled isotype-specific antibodies. Arrow indicates secondary immunization on day 30.

when the IgM production by C_3H/HeJ mice was significantly greater than that of the other strains.

Table XXVI shows the ratios of gamma isotypes produced by the *xid*-bearing strains relative to the C_3H/HeJ strain at two time points from both the primary and secondary responses. Most notably, the primary and secondary IgG_3 and the secondary IgG_1 responses were reduced in the *xid*-bearing strains. The ratios of IgG_{2b} levels were somewhat decreased in the primary response, though levels in the secondary response were approximately the same. The ratios of IgG_{2a} levels were variously increased or decreased at different time points.

2. PR8 VIRUS SPECIFIC ANTIBODY RESPONSE AFTER AEROSOL INFECTION

In a separate experiment mice from the CBA/N, C_3H/HeJ, C_3H/HeN and C_3HeB/FeJ strains underwent aerosol infection with a PR8 virus suspension containing 3×10^5 PFU/ml. The mice were sacrificed 21 days post-infection, and isotype profiles of PR8 virus-specific antibodies were determined for individual serum specimens(Table XXVII). The IgG_3 and IgG_1 responses of the CBA/N strain were depressed relative to responses in the three strains that do not bear the *xid* defect. These results are consistent with those observed following intraperitoneal immunization(Table XXVI). There were no consistent

Table XXVI. Ratios of gamma subclasses produced after parenteral immunization by Lyb5$^-$ strains(CBA/N and C_3J.*xid*) relative to the Lyb5$^+$ strain(C_3H/HeJ)

	CBA/N:C_3H/HeJ			
	IgG3	IgG1	IgG2b	IgG2a
10 Days[a]	0.13	--[b]	0.30	3.33
20	0.16	--	0.47	8.33
40	0.33	<0.06[c]	1.05	0.19
50	0.47	<0.06	0.59	0.67
	C_3J.*xid*:C_3H/HeJ			
	IgG3	IgG1	IgG2b	IgG2a
10	0.01	--	0.17	0.60
20	0.21	--	0.40	2.40
30	0.22	<0.06	0.67	1.94
40	0.11	<0.06	1.25	0.52

[a]As in Figures 14 and 15 mice underwent parenteral immunization on days 0 and 30. Ratios of concentrations of subclasses in sera of Lyb5$^-$ strains relative to the concentration in sera of the Lyb5$^+$ strain at 2 time points of the primary response(10 and 20 days) and 2 of the secondary response(40 and 50 days) are presented here.

[b]No detectable response in any strain.

[c]Because there was no detectable response in the Lyb5$^-$ strains, the sensitivity of the assay(~0.1 microgram/ml) was divided by the Lyb5$^+$ value to arrive at the ratio.

Table XXVII. Concentrations of PR8 virus-specific antibodies of different isotypes following aerosol infection[a]

Isotype	CBA/N	C_3H/HeJ	C_3H/Hen	C_3HeB/FeJ
IgM	88(49)[b]	103(45)	162(91)	29(14)
IgG3	2.4(1)	32(9)	33(11)	35(8)
IgG1	9(2)	17(3)	14(4)	24(7)
IgG2b	83(19)	23(2)	56(13)	49(13)
IgG2a	300(155)	544(174)	315(117)	120(54)
IgA	29(9)	18(6)	2(2)	13(7)

[a]Mice underwent aerosol infection with a virus suspension containing 3 x 10^5 PFU/ml. Twenty-one days post-infection mice were sacrificed, bled and individual sera were employed in the solid phase radioimmunoassay isotype determination.

[b]Micrograms/ml(SEM)

differences among the strains with regard to the IgM and IgG_{2a} isotypes.

IgG_{2b} levels in CBA/N mice were somewhat greater than those of normal mice, the reciprocal of the relationship with the IgG_3 and IgG_1 isotypes(Table XXVII). The IgG_{2b} differences were viewed as less noteworthy because they were not confirmed by the results of the parenteral immunization(Table XXVI). IgA levels were increased in CBA/N mice relative to normal strains in this experiment.

As was the case with parenteral immunization, the kinetics of HI antibody responses following aerosol infection of the different strains of mice were not distinguishable(data not shown). Moreover, the severity of infection as assessed by peak pulmonary virus titers and the extent of pneumonia following infection were virtually identical in all groups(data not shown).

IX. DISCUSSION

A. DIVERSITY OF MONOCLONAL ANTIBODIES SPECIFIC FOR SEQUENTIAL PR8 VIRUS VARIANTS

The diversity of the anti-influenza B cell repertoire has been examined in a system in which a panel of viruses -- the parental PR8 virus and sequentially selected variants -- has a corresponding panel of monoclonal antibodies that are specific for the distinguishing antigenic determinant of each virus. That is, monoclonal antibodies have been generated that are specific for the antigenic alteration that distinguishes the members of the virus panel.

The central question is what diversity generating mechanisms are called into play when a combining site is required for a new epitope that reflects a single amino acid alteration. The hypothesis is based on previous findings in this system(Liu et al., 1981; Liu et al., 1983; Moran et al., 1984) of extensive cross reactive idiotypy among monoclonal antibodies from different fusions that are directed to distinct epitopes. Consequently, one might argue that monoclonal antibodies specific for sequential virus variants might be even more likely to share an idiotype. Such monoclonal antibodies might have a somewhat restricted genetic origin and consequently somatic events would be particularly important in generating their paratypic diversity.

The idiotype defined by the rabbit antiserum to PY102 is borne by 3 of the 4 sequential virus variant-specific monoclonal antibodies -- PY102, VM113 and VM202(Table VIII). This idiotype is relatively independent of light chain since VM202 is V_K21^-, while PY102 and VM113 both use V_K21 subgroups A, D, E or F(Figure 3). Most importantly, there is a strong association between this IdX and the 7183 V_H family. All of the monoclonal antibodies tested that express a 7183 V_H gene -- PY102, VM202, PT109 and XY101 -- are IdX^+. Monoclonal antibodies that use the S107, J558 and 36-60 V_H families are IdX^-(Table VIII). Idiotopes defined on PY206 and PY211, both V_H.J558, are not seen on the monoclonal antibodies that bear the PY102 IdX(Tables XII-XV). It is important to point out that the 5 IdX^+ monoclonal antibodies -- PY102, VM113, VM202, PT109 and XY101 -- are from 5 different mice and are individually distinct in terms of paratype. Specifically, PY102, VM113 and VM202 are directed to the H1 hemagglutinin, while XY101 is directed to the H3 hemagglutinin and PT109 to the HA-2 portion of the H1 hemagglutinin.

VM201 stands apart from the other sequential variant-specific monoclonal antibodies in that it is idiotypically distinct. The VM201 IdX is seen on H36-5-3 and H36-7-3 -- monoclonal antibodies specific for the Sb antigenic region of the PR8 hemagglutinin(Staudt and

Gerhard, 1983) -- in a sandwich assay(Table X), but only H36-5-3 in a competitive RIA(Table XI). This probably reflects the relative sensitivities of the assay systems. As is the case with the PY102 IdX, the light chain is not strongly involved in expression of this idiotype because H36-5-3 and H36-7-3 use the V_K21 C subgroup while VM201 uses the A, D, E or F subgroup. Most importantly, these 3 monoclonal antibodies all use the S107 family of V_H genes. Monoclonal antibodies that utilize the 7183 and J558 V_H gene families do not express this IdX, nor do 11 PC binding monoclonal antibodies that also use a member of the S107 gene family(Tables X and XI). Idiotopes on PY206 and PY211(Tables XII-XV), both V_H J558$^+$, are not seen on VM201, H36-5-3 and H36-7-3.

An explanation for the absence of the IdX on PC binding monoclonal antibodies that use S107 V_H genes may be that they use a different member of the 4 member S107 family than the IdX$^+$ monoclonal antibodies. In fact, Clarke et al.(1985) have shown that his S107$^+$, hemagglutinin-specific monoclonal antibodies use the V11 germ line member of the S107 family, while PC binding myeloma proteins use the V1 member(Crews et al., 1981). It is also possible that somatic events subsequent to antigen stimulation may contribute to the presence or absence of the Ids. We conclude that the S107 V_H family is necessary, though not sufficient for expression of the

VM201 IdX.

The association between the PY102 IdX and the 7183 V_H family and the VM201 IdX and the S107 V_H family directly demonstrates a long held assumption that cross reactive idiotypes are markers of germ line genes. Though this has been shown repeatedly in haptenic systems(Crews et al., 1981; Siekevitz et al., 1983; Bothwell et al., 1981; Clevinger et al., 1980), this relationship has not been demonstrated before in the anti-influenza immune response.

Knowledge of the idiotypy and V genes used in these monoclonal antibodies specific for sequentially selected PR8 virus variants allows for an accounting of their paratypic diversity. Both germ line encoded diversity and somatic diversifying mechanisms may have played a role.

VM201 and VM202 clearly demonstrate germ line encoded diversity, particularly because they might have suggested a role for somatic mutational events in that they are from the same fusion and can only be distinguished paratypically after extensive selection of variant viruses(Table VII). However, they are not idiotypically similar(Tables VIII, X and XI). They also use different V_H gene families(Figures 4 and 5) and different V_K groups(Figure 3). Clearly, the diversity residing in the germ line accounts well for the paratypic

differences between these 2 monoclonal antibodies.

On the other hand, the relative similarity of VM201 and VM202, in that they are difficult to distinguish on a panel of variant viruses, may offer a role for somatic events. This speculation could only be verified by comparison of their sequences with germ line sequences.

PY102 and VM113, though they have reciprocal specificities(Tables IV and V), are quite similar in other respects. They both use V_K21 light chains of the A, D, E or F subgroups(Figure 3). Though we have not shown directly that they use the same V_H family, they do share an idiotype that is strongly associated with the V_H 7183 family. Given the caveat that we do not yet know the nature of the D, J_H and J_L genes, the genetic similarities of these paratypically distinct monoclonal antibodies suggests a role for somatic mutational events in generating their distinct combining sites.

The V_K21 group of light chains is highly represented -- 3 of the 4 monoclonal antibodies specific for sequential PR8 virus variants are V_K21^+(Figure 3). Previous analyses of anti-influenza monoclonal antibodies have shown that 22%(Staudt and Gerhard, 1983) and 47%(Moran et al., manuscript in preparation) are V_K21^+, an inordinately high percentage since only 8.6% of normal BALB/c K-bearing serum immunoglobulin is V_K21^+(Julius et al., 1981).

Recently, Clarke et al.(1985) showed that use of the V_K21C subgroup is strongly associated with monoclonal antibodies directed to 1 of the 4 major antigenic regions of the hemagglutinin -- the Sb region(Gerhard et al., 1981). Somatic mutational events in the V_K21C subgroup light chain were shown to be very important for the fine specificity. No particularly important contribution of a V_K21 light chain to fine specificity is apparent here, since the 2 most paratypically similar monoclonal antibodies -- VM201 and VM202 -- do not both use a V_K21 light chain.

Presently, all the V_H families except J606 are known to be used in the anti-influenza response(Moran et al., manuscript in preparation; Clarke et al., 1985). Though we have as yet determined the V_H gene family used by only 12 monoclonal antibodies, 5 of the 12 or 42% use the V_H 7183 family. This is somewhat surprising because the 7183 germ line genes represent only 11% of the total estimated V_H genes(Brodeur and Riblet, 1984).

Our ability to generate monoclonal antibodies, such as VM113, that bind much more avidly to the variant(PY102-V1) than to the parental virus(Tables IV and V) establishes that a new epitope is present on the variant virus. Alternatively, the epitope recognized by PY102 might have been lost without a novel epitope having been produced. In contrast, our inability to identify a

monoclonal antibody specific for the VM113-V10 variant virus, despite a very productive fusion in which many other influenza virus specific monoclonal antibodies were generated, could indicate that in that variant mutational events may have resulted in the loss of an epitope without a new one being generated. An alternative possibility, as has been postulated to explain the lack of exact correlation between the antigenic regions of the H1 and H3 hemagglutinins(Caton et. al., 1982), is that the addition of a new glycosylation site made an epitope inaccessible. Moreover, Skehel et al.(1984) have shown that a carbohydrate side chain of the Hong Kong hemagglutinin inhibits recognition by a monoclonal antibody. It should be made clear, however, that the inability to produce a monoclonal antibody specific for the VM113-V10 virus by no means proves the hypothesis that an antigenic determinant has been lost or hidden.

This demonstration of antigenic change by means of the gain of an epitope or the possible loss of an epitope concurs with findings of Webster et al.(1983) in which he utilized polyclonal sera that had been thoroughly adsorbed on the parental virus rather than variant specific monoclonal antibodies. Webster et al.(1983) also demonstrated on the amino acid level that a second generation variant virus may be a revertant to the original parental virus -- a possible explanation for

VM113-V1 and the other "PR8-like" variants(Table III).

Distinguishing the fine specificities of VM201 and VM202 by further sequential selection of virus variants was informative(Tables VI and VII). Firstly, it indicated that though a single amino acid change in the hemagglutinin is often sufficient to differentiate 2 combining sites, here sequential selection of amino acid changes affecting a single antigenic region was necessary in this case. Secondly, it underlined the inherent limitations of using a restricted panel of variant viruses in estimating the size of the B cell repertoire.

B. ANTI-IDIOTYPES AS IMMUNOGENS

The approach to eliciting an anti-influenza response by immunization with anti-idiotype reagents can be viewed as 2 semi-independent projects. Firstly, it was necessary to thoroughly characterize idiotope bearing antibodies in terms of antigen specificity and also the kinetics of their expression throughout the immune response. This provides a rational foundation for the major project -- planning and interpreting attempts to successfully immunize with anti-idiotope reagents.

Our interest in anti-idiotype immunization received a great deal of impetus from the demonstration with syngeneic, polyclonal anti-PY206 idiotype antibodies that monoclonal antibodies directed to both the H1 hemagglutinin of PR8 virus and the H3 hemagglutinin of X-

31 virus share an idiotype(Moran et al., 1984). This finding offered the enticing theoretical possibility of using anti-idiotypes to elicit an anti-influenza response that is cross reactive for different hemagglutinin subtypes. A panel of monoclonal anti-idiotopes was generated to provide well characterized reagents for these studies(Moran et al., manuscript in preparation).

1. CHARACTERIZATION OF MONOCLONAL ANTI-IDIOTOPE REAGENTS

Characterization of the PY206 idiotopes(defined by the monoclonal anti-idiotopes SN3-1A, SN3-9A and SP3-5A) and the PY211 idiotope(defined by the monoclonal anti-idiotope 63-4) was done in the most relevant system -- immune sera. We first looked at sera from BALB/c mice undergoing a secondary response to PR8 and X-31 viruses. This showed that the idiotopes defined by SN3-9A and SP3-5A are clearly used in the responses to both viruses(Figure 12), strongly confirming the earlier findings with polyclonal anti-PY206-idiotype antibodies.

The findings with immune sera require demonstrating that the idiotope bearing antibodies are also virus binding. Antibodies of unknown specificity that share an idiotype with a given antibody -- parallel sets -- are not uncommon. Indeed, this has been clearly demonstrated in the immune response to B/Lee virus(Liu et al., 1981).

The most rigorous demonstration that an idiotope defines an antibody with true viral specificity is by

adsorption studies. The extremely high idiotope titers of serum 103 made this possible. These studies indicated not only that the SN3-9A and SP3-5A defined idiotopes are primarily on virus binding antibodies because removal of PR8 virus binding antibody diminishes idiotope expression, but also that these idiotope bearing antibodies are HA and/or NA specific since removal of X-31 virus binding antibody does not affect idiotope expression(Figures 15-20).

We also looked at the responses to a wider range of viruses -- B/Lee, X-31(H3N2), PR8(H1N1), A/Singapore(H2N2), A/Houston(H1N1) and A/Chicken. Though the idiotopes defined by SN3-1A, SN3-9A and 63-4 were not significantly represented in any sera in this experiment, the SP3-5A defined idiotope was seen in all 6 responses, including the response to B/Lee virus(Figure 14).

Tentative conclusions can be drawn concerning the virus binding ability of idiotope$^+$ serum antibody by comparing the kinetics of HI antibody and idiotope expression during the response. A large increase in idiotope expression that is not accompanied by a comparable increase in HI titer would suggest the existence of a parallel set. This, however, is not the case in the immune responses to the 6 different influenza viruses(Figures 13 and 14). The general increase in inhibitory activity in the SP3-5A system up to day 37 is

roughly accompanied by increases in the HI titer. Therefore, in these 6 systems the SP3-5A defined idiotope appears to be largely residing on HI antibody.

Though the adsorption data is derived from the serum(103) of an individual mouse, in conjunction with comparisons of the kinetics of HI titers and idiotope expression(Figures 13 and 14) in the immune responses to 6 different influenza viruses it appears that the SN3-9A and SP3-5A defined idiotopes predominantly reside on virus binding antibody. This is an absolutely essential prerequisite to anti-idiotope immunization. Clearly, there is no point, and even potential harm, in eliciting non-virus binding antibody of unknown specificity. The hemagglutinin and/or neuraminidase specificity of the idiotope bearing antibodies in serum 103 is even more pertinent because neutralizing antibody is predominantly hemagglutinin specific(reviewed by Schulman, 1975)

The reciprocal question concerning the proportion of virus binding antibody that is idiotype^{+} was answered for serum 103 by adsorption on microtiter plates coated with SP3-5A anti-idiotope antibodies(Figure 21). This adsorption clearly had no detectable effect on total virus binding antibody(Figure 22) or HI antibody(Figure 23), indicating that this idiotope represents a minor component of the antiviral response. This experiment was important because it showed that despite the extremely

high titer of idiotope^{+} antibody in this serum(Table XVII), idiotope^{-} antibody still constitutes the vast majority of the virus specific and HI antibody responses. These results suggest that experiments designed to study the effects of idiotype suppression are unlikely to provide evidence of any effect on the total response.

We showed in Table XIX that the SN3-1A, SP3-5A and 63-4 defined idiotopes are also used by human monoclonal anti-influenza antibodies. However, several rabbit sera and a single goat anti-influenza serum did not contain detectable quantities of these idiotopes(data not shown). It has been suggested that an anti-idiotope that reacts with antigen specific antibody from different species is likely to bear a conformational structure that mimics antigen and hence represents a true internal image of antigen (beta anti-idiotope). This concept, however, has serious limitations because not all antigenic determinants may be equally immunogenic in all species and also because of the idiosyncratic nature of the immune response even among genetically identical animals. Hence, the absence of antibody in polyclonal antiviral sera of animals of different species which react with the anti-idiotope reagents employed clearly does not exclude the possibility that one or more of the anti-idiotopes may bear a conformational structure resembling a structure on the hemagglutinin.

2. ANTI-IDIOTOPE IMMUNIZATIONS

New attempts to elicit an antiviral immune response by immunizing with monoclonal anti-idiotopes are currently ongoing. We have adopted 2 important new approaches:

i. Because all of the 4 idiotopes are expressed at higher levels in secondary antiviral responses relative to the primary response(a fact not known when the first experiment was initiated), groups of mice have received more than a single injection of the anti-idiotope prior to the viral immunization.

ii. Because it is very difficult to elicit T cell help in a syngeneic animal, we have also utilized C_3H/HeJ mice in this experiment. It is certainly fair to take advantage of allotypic differences, since that will be the case in any attempts to use anti-idiotypes as vaccines in humans. We have also included groups of mice that will receive the anti-idiotope coupled to KLH(Keyhole Limpet hemocyanin).

C. ISOTYPE PROFILES OF ANTI-INFLUENZA ANTIBODIES IN MICE BEARING THE XID DEFECT.

It has been reported previously that HI antibody was not detectable in the sera of CBA/N mice 28 days after primary immunization with Formalin-inactivated virus.

However, in the same study, it was noted that immunized CBA/N mice were resistant to lethal virus challenge(Lucas et al., 1978). In contrast, in the present study serum HI antibody titers at 4 time points in the primary response as well as the secondary response were indistinguishable from those of C_3H/HeJ mice. We cannot explain the discrepancy between our results and those of Lucas et al.(1978). However, since Formalin-inactivated virus does not elicit primary cytotoxic T cell responses in immunologically intact mice(Reiss and Schulman, 1980), the antiviral immunity noted in the earlier study and the intact HI antibody responses observed in CBA/N and C_3J.*xid* mice in our experiments are consistent with the hypothesis that the $Lyb5^+$ cell subset is not required for the production of virus specific antibody of high avidity.

However, further investigation of the isotypes of PR8 virus specific antibodies did disclose differences. The *xid* defect present in both the CBA/N and the C_3J.*xid* strains is associated with decreased levels of IgG3 and IgG1 in both the primary and secondary responses relative to the levels of those isotypes in 3 strains that do not bear the *xid* defect(Tables XXVI and XXVII). Most importantly, one of the normal strains, C_3H/HeJ, is congenic to the C_3J.*xid* strain(Mond et al., 1983). It should also be emphasized that in the case of the sera of

aerosol-infected mice, reduced levels of IgG1 and IgG3 were observed in individual serum specimens, thus emphasizing the reproducibility of these results.

Given that the *xid* defect effects an inability to respond to certain carbohydrate antigens, one interpretation of the isotype pattern may be that the IgG3 and IgG1 levels are decreased because these subclasses are predominantly involved in responses to TI-2 carbohydrate antigens. However, comparison of results of solid phase radioimmunoassays in which wells were coated with egg grown virus, the immunogen in all these experiments, or with virus grown in Madin-Darby canine kidney cells, showed no differences(data not shown). This indicates that at best only a minor component of the response was specific for host carbohydrate.

Two basic isotype patterns have emerged in the response of the *xid*-bearing strains to thymus-dependent antigens(Press, 1981). A significant reduction of IgG3 with slight reductions in IgM and IgG1 has been seen in response to DNP-Hy(2,4-dinitrophenyl conjugated to *Limulus polyphemus* hemocyanin)(Press, 1981). This is analogous to the response to TI-1 antigens and is most similar to our results with influenza virus. A second pattern, which is considered analogous to the TI-2 response, shows a reduction in all isotypes. This has been seen with (T,G)-A--L(Press, 1981), sheep

erythrocytes(Phillips and Campbell, 1982) and a thymus-dependent form of B512 dextran(Mongini et al., 1981).

In the present experiments differences in isotype production were not reflected in differences in susceptibility to influenza virus infection. In other experiments(data not shown) it was observed that the CBA/N and C_3J.*xid* strains did not differ from the non-defective strains in terms of 50% lethal dose, lung lesions, lung virus titers or lung interferon production after aerosol infection. These results are consistent with those of Lucas et al.(1978), who also observed no differences in mortality of CBA/N and Lyb5$^+$ CBA/CaJ mice after challenge.

X. SIGNIFICANCE

A. The paratypic diversity of a panel of monoclonal antibodies that are specific for a corresponding panel of sequentially selected influenza virus variants can be accounted for in terms of both germ line encoded and somatically generated diversity. Two cross reactive idiotypes have been defined that are associated with the 7183 and S107 V_H gene families, respectively. These monoclonal antibodies also appear to preferentially use V_K21 light chains.

B. Monoclonal anti-idiotope antibodies to influenza specific monoclonal antibodies define serum idiotope bearing antibodies that are expressed in the immune response to a variety of influenza viruses and that also appear to be hemagglutinin and/or neuraminidase specific.

C. Mice bearing the *xid* defect generate a humoral response to influenza virus that is deficient in the IgG3 and IgG1 isotypes. This is not reflected in an increased susceptibility to influenza infection.

XI. APPENDIX: ABBREVIATIONS

BSA - bovine serum albumin

DEAE - diethylaminoethyl

DMSO - dimethyl sulfoxide

EBV - Epstein-Barr virus

FCA - Freund's complete adjuvant

FCS - fetal calf serum

FIA - Freund's incomplete adjuvant

HA - hemagglutinin glycoprotein

HA-1 - hemagglutinin amino terminal cleavage product

HA-2 - hemagglutinin carboxy terminal cleavage product

HAT - hypoxanthine-aminopterin-thymidine

HI - hemagglutination inhibition

H1N1 - hemagglutinin and neuraminidase subtypes

H2N2 - hemagglutinin and neuraminidase subtypes

H3N2 - hemagglutinin and neuraminidase subtypes

IdI - individual idiotype

IdX - cross reactive idiotype

M_1 - influenza virus matrix protein

M_2 - influenza virus non-structural protein derived from RNA segment 7

NS_1 - influenza virus non-structural protein derived from RNA segment 8

NS_2 - influenza virus non-structural protein derived from RNA segment 8

PA - acidic polymerase protein of influenza virus

PB1 - the larger of the 2 basic polymerase proteins of influenza virus

PB2 - the smaller of the 2 basic polymerase proteins of influenza virus

PBS - phosphate buffered saline

PC - phosphorylcholine

PEG - polyethylene glycol

PFU - plaque forming unit

PR8 virus - influenza A/PR/8/34 virus(H1N1)

PY102-V1 - a variant of PR8 virus selected with the monoclonal antibody PY102

RDE - receptor destroying enzyme

RIA - radioimmunoassay

RNP - ribonucleoprotein

SP2/0 - BALB/c myeloma cell line

TI-1 - thymus independent antigen type 1

TI-2 - thymus independent antigen type 2

V_H - heavy chain variable region

V_L - light chain variable region

VM113-V1 - a variant of PY102-V1 virus selected with the monoclonal antibody VM113

X-31 virus - a reassortant influenza virus [(H3N2) x (H1N1)]

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