

IDIOTYPY OF CLONAL RESPONSES TO INFLUENZA VIRUS HEMAGGLUTININ*

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The structural basis for the genetic and antigenic variation in influenza viruses has been extensively studied in the past to elucidate the mechanisms and requirements for the emergence of antigenically novel strains (1-4). In particular, monoclonal antibodies have been widely used to study the frequencies of antigenic variants (5-7) and to map the amino acid changes in the antigenically relevant portions of the hemagglutinin (HA)¹ molecules of natural and laboratory-obtained variants (8, 9). With the availability of primary sequence information for several different strains within the H3N2 subtype, Wiley et al. (10) have identified four antigenic sites on the three-dimensional structure of Hong Kong virus HA molecules using x-ray crystallography.

In contrast to the impressive advances that have been made in the understanding of structure and variation of influenza virus HA, only limited data are available related to clonal expression during anti-HA responses. Cancro et al. (11) compared the reactivity pattern of monoclonal anti-HA antibody populations in 12-14-d-old and adult mice, and obtained preliminary results indicating that there were relatively few responding clonotypes in young animals as compared with adult animals.

The aim of the present study was to investigate the clonal basis of immune responses to influenza viral HA by studying the idiotype of monoclonal antibodies and anti-HA antibodies synthesized in response to immunization with influenza virus. Idiotypes have been widely used as tools to study clonal responses to a variety of antigens, taking advantage of the fact that idiotypes are phenotypic markers of V region genes (12). Our results showed that monoclonal antibodies specific to different determinants of PR8 HA share idiotypes and that monoclonal antibodies specific to the same or overlapping determinants of B/Lee HA also share idiotypes. The cross-reactive idiotypes of monoclonal antibodies are shared with anti-HA antibodies synthesized in response to a conventional immunization with intact B/Lee virus. Variations and persistence of the cross-reactive idiotypes were observed during conventional primary and secondary anti-HA antibody responses.

Materials and Methods

Mice. BALB/c mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and were used between 5 and 15 wk of age.

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¹ *Abbreviations used in this paper:* FCS, fetal calf serum; HA, hemagglutinin; HI, hemagglutination inhibition; IdI, individual idiotype; IdX, cross-reactive idiotype; RDE, receptor-destroying enzyme; RIA, radioimmunoassay; SRBC, sheep erythrocytes; SRIA, solid-phase radioimmunoassay.

Monoclonal Antibodies. Monoclonal antibodies against HA of influenza viruses A/PR/8/34 (HON1) (PR8 virus) and B/Lee/40 (B/Lee virus) raised by M. D. Lubeck et al. (6) were used.

Viral Antigens. Influenza PR8 virus and B/Lee virus were obtained from laboratory stocks and grown in the allantoic cavity of 10–11-d-embryonated chicken eggs and were purified by standard procedures (13).

Selection of Antigenic Variants. Antigenic variants were selected on MDCK cell monolayers using cloned virus seeds in the presence of excess monoclonal antibodies (6) to ensure that all nonneutralized virus plaques were produced by variants. Each plaque was picked separately and was confirmed to be a variant by antiviral hemagglutination inhibition (HI) test and/or the absence of neutralization with the same monoclonal antibody used in selection. Seed stocks of the variants were prepared by inoculation of 10–11-d-embryonated chicken eggs (13).

Determination of Subclasses. Subclasses of monoclonal antibodies were determined by solid-phase radioimmunoassay (SRIA) (14). Briefly, microtiter wells were coated with 50 μ l of 50 μ g/ml of affinity-purified monoclonal antibodies overnight. After three washings with saline, they were incubated for 1 h with 50% fetal calf serum (FCS), and then with different 3 H-labeled goat antimurine IgM or IgG classes for 3 h. After extensive washing, the radioactivity was counted in a scintillation counter. The 3 H-labeled goat antimurine Ig antibodies were a gift from P. Mongini (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Preparation of Anti-idiotypic Antisera. Syngeneic anti-idiotypic (anti-Id) antisera against monoclonal antibodies B118, B142, and B147 (specific for B/Lee HA), and homologous anti-Id antiserum against monoclonal antibody P28 (specific for PR8 HA) were prepared in BALB/c and A/J mice, respectively, according to a previously described technique (15). The homologous anti-Id antiserum was extensively adsorbed on a Sepharose 4B-BALB/c Ig column to eliminate antiallotype antibodies before use. (Initial efforts to raise syngeneic anti-P28 antiserum were unsuccessful.)

Immunization and Bleeding. 24 mice were divided into two groups. In the primary immune response group, mice were immunized with 5 μ g of purified B/Lee virus and bled 5, 10, 20, 30, and 60 d after immunization. In the secondary immune response group, mice primed with 5 μ g of purified B/Lee virus were boosted 1 mo later with the same amount of B/Lee virus, and then bled 3, 7, 10, 20, and 30 d after boost. Antiviral HA titers of antisera pooled from pairs of mice in the group of 12 mice were measured in antiviral HI assays, and were reported as means \pm SD. The expression of cross-reactive idiotypes was studied by HI assays and radioimmunoassay (RIA).

Antiviral HI Assays. Sera or ascites fluids were assayed in a standard antiviral HI test after treatment with receptor-destroying enzyme (RDE, World Health Organization, Atlanta, Ga.; 16). The HI titers were reported as \log_2 of the reciprocal of the highest dilutions of RDE-treated samples that inhibited hemagglutination.

Assays for Idiotypes. The assays for cross-reactive idiotypes involved HI and RIA, and were performed as follows. (a) HI assay (15): sheep erythrocytes (SRBC) were coated with 1 mg/ml purified monoclonal antibodies using the chromic chloride method (17). The capacity of other monoclonal antibodies or antiviral sera to inhibit agglutination of these coated erythrocytes by the corresponding or noncorresponding anti-Id antibody was determined. The HI titers were reported as \log_2 of the reciprocal of the highest dilution of inhibitors (original concentration = 1 mg/ml) that inhibited hemagglutination. (b) RIA (18): both direct binding and competition inhibition assays were used to study cross-reactive idiotypy. Microtiter wells were coated with affinity-purified anti-Id sera by overnight incubation followed by three washings with saline. They were then incubated with 50% FCS for 1 h and washed three times. In direct binding assays, plates were incubated with 125 I-labeled monoclonal antibodies for 3 h. After extensive washing, the radioactivity was counted in the γ -spectrophotometer. In competition assays, measurements were made of the capacity of other monoclonal antibodies to inhibit binding of 125 I-labeled monoclonal antibody to anti-Id antibody-coated microtiter plates.

Viral Inhibition Assay. To determine the ability of purified virus to inhibit the binding of monoclonal antibodies with anti-Id antibodies, mixtures of 125 I-labeled monoclonal antibodies and various concentrations of purified virus were preincubated for 1 h and then added to plates coated with anti-Id antibodies.

Iodination of Monoclonal Antibodies. Affinity-purified monoclonal antibodies were iodinated according to chloramine T method described by Greenwood et al. (19). The specific activities were $\sim 15 \mu\text{Ci}/\mu\text{g}$ protein. The labeled monoclonal antibodies were stored at -20°C in aliquots and used within 6 wk of iodination.

Results

Characterization of Monoclonal Antibodies to PR8 Virus in Terms of Antiviral HI Titers, Subclasses, and Fine Specificities. The monoclonal antibodies to PR8 virus HA used in these studies were P8, P20, and P28. Their antiviral HI titers and subclasses are listed in Table I. All three monoclonal antibodies have similar antiviral HI activity. P8, P20, and P28 belong to subclasses IgG_{2b} , IgG_{2b} , and IgG_1 , respectively.

These monoclonal antibodies were determined to be specific for distinct antigenic determinants on PR8 HA on the basis of the following criteria. (a) The frequencies of antigenic variants in the presence of pairs of monoclonal antibodies were equivalent to the products of those observed in the presence of each monoclonal antibody alone (5) (data not shown). (b) When variants selected in the presence of a particular

TABLE I
Summary of Antiviral HI Titers and Subclasses of Monoclonal Antibodies

Monoclonal antibodies	Antigen recognized	HI titers (\log_2 units)*	Subclasses
P8	PR8 (HA)	7	IgG_{2b}
P20	PR8 (HA)	8	IgG_{2b}
P28	PR8 (HA)	8	IgG_1
B109	B/Lee (HA)	7	IgG_1
B118	B/Lee (HA)	8	IgG_1
B123	B/Lee (HA)	9	IgG_1
B127	B/Lee (HA)	8	IgG_1
B141	B/Lee (HA)	8	IgG_1
B142	B/Lee (HA)	7	IgG_{2a}
B147	B/Lee (HA)	9	ND‡

* Twofold dilutions of RDE-treated ascites were used to inhibit the hemagglutination of human erythrocytes by 32 hemagglutinating units of either PR8 or B/Lee virus. The titers are expressed as the reciprocal (\log_2) of the highest dilution that causes inhibition of hemagglutination.

‡ Not determined.

TABLE II
Reactivity of Monoclonal Antibodies to PR8 Virus HA with Variants Selected with Different Monoclonal Antibodies

Viruses	Number of variants examined	Range of HI titers (\log_2 units)*		
		P8	P20	P28
PR8	—	7	8	8
P8 variants‡	8	<1	6 ~ 7§	6 ~ 8
P20 variants‡	7	5 ~ 7	<1	6 ~ 8
P28 variants‡	6	5 ~ 7	6 ~ 7	≤ 1

* Same as Table I, except PR8 viruses or variants were used.

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

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

monoclonal antibody were examined in HI or neutralization assays, monoclonal antibodies directed to other determinants still reacted at relatively unchanged titer. The HI titers of P8, P20, and P28 to PR8 virus and variants are shown in Table II. It can be seen that variants selected in the presence of P8 (P8 variants), in the presence of P20 (P20 variants), or in the presence of P28 (P28 variants) did not react with the monoclonal antibodies used in selection. However, the titers of monoclonal antibodies not used in the selection remained relatively unchanged. For instance, P8 no longer reacted with P8 variants but P20 and P28 still reacted with P8 variants at high titers. Thus, it is evident that P8, P20, and P28 are reactive with different determinants on the PR8 HA molecule.

Characterization of Monoclonal Antibodies to B/Lee Virus HA: HI Titers, Subclasses, and Fine Specificities. The HI titers and subclasses of the monoclonal antibodies to B/Lee HA are also listed in Table I. Again, these monoclonal antibodies have similar HI titers, and B109, B118, B123, B127, and B141 belong to subclass IgG₁, but B142 belongs to subclass IgG_{2a}.

These monoclonal antibodies were determined to be directed against the same or overlapping epitopes partially on the basis of HI results shown in Table III. In all cases, monoclonal antibodies used to select variants either did not react with those variants or reacted at significantly lower titer than with wild-type virus. In addition (Table III), monoclonal antibodies other than those used to select a particular group of variants did not react or reacted at significantly lower titer with at least some of the variants in that group than with wild-type virus. For example, all monoclonal antibodies tested reacted at lower titer with all six variants selected in the presence of B118 than with wild-type virus. Similar results were obtained in HI assays with at least some of the variants selected with other individual monoclonal antibodies. Although other monoclonal antibodies reacted with the two variants selected in the presence of B147 at relatively high titer, B147 reacted at reduced titer with all but a few of the variants selected in the presence of other monoclonal antibodies, strongly suggesting that B147 is directed at a determinant that overlaps with that recognized

TABLE III
Reactivity of Monoclonal Antibodies to B/Lee Virus HA with Variants Selected with Different Monoclonal Antibodies

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

* Same as Table I, except that either B/Lee virus or variants were used.

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

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

TABLE IV
*Reactivity of Monoclonal Antibodies to PR8 Virus HA with A/J Anti-P28
 Antiserum Assayed by HI and RIA*

Monoclonal antibodies	HI titers (log ₂)*	Competition RIA (purified antibodies giving 50% inhibition)‡
		<i>μg/ml</i>
P8	6	<0.01
P20	>12	<0.01
P28	6	<0.01
B109	0	>100

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

‡ Concentration of purified monoclonal antibody that causes 50% reduction of binding of ¹²⁵I-labeled P28 (50,000 cpm of ~10⁶ cpm/μg) to 20 μg/ml anti-P28-coated wells.

TABLE V
*Inhibition by PR8 Virus of Binding of Monoclonal Antibodies against PR8
 HA to Anti-P28Id Antibody*

¹²⁵ I-labeled monoclonal antibodies	Purified PR8 virus giving 50% inhibition*
	<i>μg/ml</i>
P8	0.9
P20	0.8
P28	0.4

* Concentration of purified PR8 virus that causes a 50% reduction of binding of ¹²⁵I-labeled monoclonal antibodies (50,000 cpm of 10⁵ cpm/μg) to 40 μg/ml anti-P28Id antibody-coated wells.

by the other antibodies. In addition, when these variants were tested in neutralization assay with the monoclonal antibodies listed in Table III, reduced reactivity was observed in all instances (data not shown). Finally, the frequency of antigenic variants detected in cloned virus seeds in the presence of a single monoclonal antibody was equivalent to that observed when any two monoclonal antibodies were combined (data not shown).

Cross-reactive Idiotypy among Monoclonal Antibodies to PR8 Virus HA. When homologous anti-P18 antiserum was used to detect shared idiotypes among P8, P20, and P28, extensive reactivity was demonstrated (Table IV) in both HI assays and competition RIA. These results were somewhat surprising in view of the fact that we had previously demonstrated that these three monoclonal antibodies are directed to different antigenic determinants. In contrast, no cross-reactivity with monoclonal antibodies to B/Lee HA was observed. Similarly, in assays of direct binding of ¹²⁵I-labeling P8, P28, and B109 (50,000 cpm of ~10⁶ cpm/μg) to anti-P28 antibody (20 μg/ml)-coated plates, the results obtained were 1,376, 9,893, and 220 cpm, respectively.

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

TABLE VI
IdI Expressed on Different Monoclonal Antibodies to B/Lee HA Detected by HI Assays and RIA

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

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

‡ Concentration of purified monoclonal antibody that causes 50% reduction of binding of ¹²⁵I-labeled B142 (50,000 cpm of ~10⁶ cpm/μg) to 3 μg/ml anti-B142-coated wells.

§ Not determined.

Cross-reactive Idiotype among Monoclonal Antibodies to B/Lee Virus HA. The presence of individual idiotypes (IdI) and cross-reactive idiotypes (IdX) on B/Lee-specific monoclonal antibodies was studied using syngeneic anti-Id antisera (i.e., BALB/c anti-B118, anti-B142, and anti-B147). The IdI was studied in three systems (Table VI). (a) Anti-B142Id antibodies vs. B142. In this system, we found that only B109 shared the idiotypic determinants of B142 detected in both HI assays and inhibition RIA. In addition, when direct SRIA (data not shown) were done with ¹²⁵I-labeled B/Lee-specific monoclonal antibodies (50,000 cpm of ~10⁶ cpm/μg) and anti-B142Id antibody (3 μg/ml), >3,000 cpm of ¹²⁵I-B142 and ¹²⁵I-B109 were bound to the plates, whereas <400 cpm of other radiolabeled monoclonal antibodies were bound. (b) Anti-B118Id antibodies vs. B118. In this system we found that B142 and B147 share some idiotypic determinants of B118 as assessed by HI assays; (c) Anti-B147Id antibodies vs. B147. No other monoclonal antibodies studied expressed the IdI of B147. These results suggest that some monoclonal antibodies (e.g., B147) may bear a true IdI, whereas some individual idiotopes (e.g., idiotypes detected by anti-B142Id antiserum) can be shared by only few monoclonal antibodies.

To study IdX, an HI assay that allowed the investigation of shared idiotypes (IdX) and minimized the role of IdI was used. This technique was used previously (20) in studies of the IdX of a human IgM myeloma with rheumatoid activity in which IdX were initially discovered and thereafter in the study of IdX of murine myeloma proteins (21).

The IdX of monoclonal antibodies were studied by HI in three systems. (a) Inhibition of binding of ¹²⁵I-labeled anti-B118Id and anti-B142Id antibodies to SRBC-B109: as shown in Table VII, in both systems all monoclonal antibodies expressed the IdX (i.e., inhibited binding). (b) Inhibition of binding of radiolabeled anti-B147Id antibodies to SRBC-B109: in this system only three of six monoclonal antibodies

TABLE VII
IdX Expressed on Different Monoclonal Antibodies to B/Lee HA Detected by HI Assays

Inhibitors	HI titers (log ₂) anti-Id antiserum + ligand*		
	Anti-B118 + SRBC-B109	Anti-B142 + SRBC-B109	Anti-B147 + SRBC-B109
B109	4	5	6
B118	5	3	0
B123	5	3	0
B127	4	3	0
B141	4	4	4
B142	4	5	6
P8	0	0	0

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

TABLE VIII
Inhibition by B/Lee Virus of Binding of Monoclonal Antibodies to Anti-Id Antibodies

¹²⁵ I-labeled monoclonal antibodies	Purified B/Lee virus giving 50% inhibition*	
	Anti-B118	Anti-B142
B109	ND‡	0.7
B118	0.6	0.9
B142	0.9	1.0

* Concentration of purified B/Lee virus that causes a 50% reduction of binding of ¹²⁵I-labeled monoclonal antibodies (50,000 cpm of ~10⁶ cpm/μg) to 10 μg/ml anti-B118 or anti-B142 antibody-coated wells.

‡ Not determined.

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

The binding of syngeneic anti-Id antibodies to monoclonal antibodies to B/Lee HA was inhibited by purified B/Lee viral antigen (Table VIII). These results indicate that anti-Id antibody recognizes antigenic determinants on monoclonal antibodies closely associated with their antigen binding sites.

Idiotypes Expressed during the Primary and Secondary Antiviral Response of Mice Immunized with B/Lee Virus. When primary and secondary immune sera from mice immunized with B/Lee virus were tested for their antiviral HI activity, typical primary and secondary responses were observed (Fig. 1). During the primary immune response, a lag period of ~5 d was followed by increasing antiviral antibody titers that reached a peak 10–20 d after immunization and then slowly declined. During the secondary immune response, antibody titers increased after a very brief lag phase (<3 d) and reached a plateau by day 7 after booster immunization.

The same immune sera were used in four systems in HI assay to study idiotypic expression during primary and secondary immune responses. As illustrated in Fig. 2, the titer of IdX detected by anti-B118 and SRBC-B123 was 2.3 ± 0.5 before

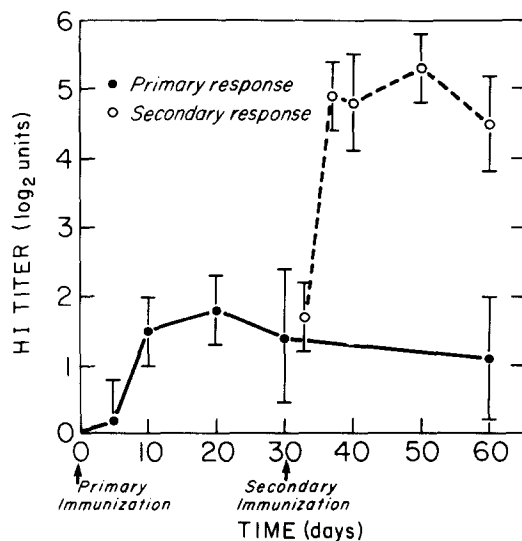


FIG. 1. Antiviral hemagglutinating inhibiting titers during primary and secondary responses of mice immunized with B/Lee virus. Titers are expressed as the geometric means of the reciprocal of the serum dilution causing inhibition of 32-64 hemagglutinating units of B/Lee virus, and are calculated from individual titrations of sera of at least six mice.

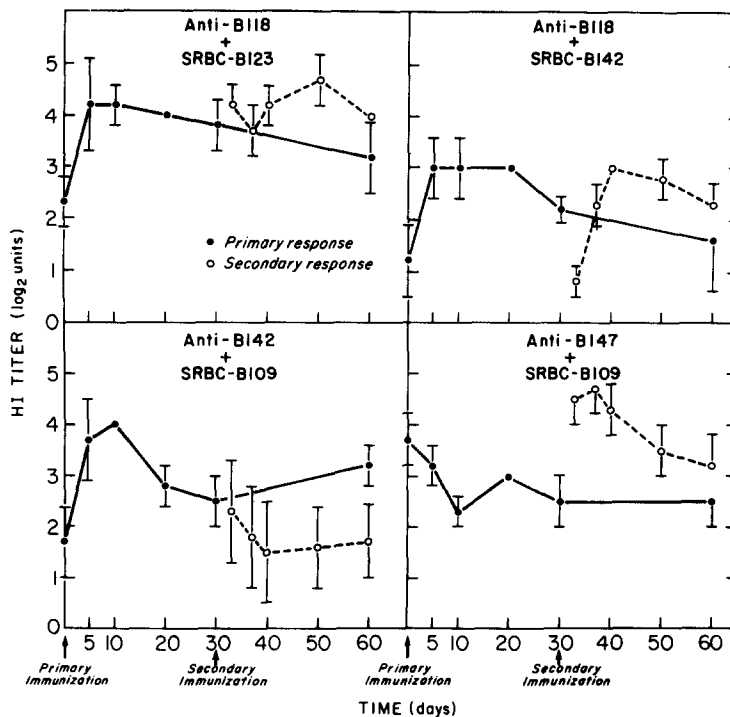


FIG. 2. IdX expressed during primary and secondary responses of mice immunized with B/Lee virus. Sera were as used in Fig. 1. Twofold dilutions of sera were used to inhibit the binding of anti-Id antisera to SRBC coated with monoclonal antibodies.

immunization, reached a peak of 4.2 ± 0.9 on day 5 during the primary immune response, and dropped gradually thereafter. Following secondary immunization, there was an initial decline in the titers of this idiotype (possibly due to formation of complexes between idiotype and viral antigen). Thereafter, titers rose slowly and reached a peak of 4.7 ± 0.5 on day 20 after secondary immunization.

The titer of IdX detected by anti-B118 and SRBC-B142 was 1.2 ± 0.7 before immunization. During the primary immune response, it reached a plateau of 3.0 ± 0.6 on day 5, and began to decline on day 20 after immunization. During the secondary immune response, the same idiotype, after an initial decline period, rose rapidly in titer, reaching a lower peak than was seen in the primary response, and declined slowly thereafter.

The HI titer of IdX detected by anti-B142 and SRBC-B109 was 1.8 ± 0.8 before immunization. After primary immunization, it rose immediately, reached a peak of ~ 4.0 between days 5 and 10, and then declined. In contrast to the other idiotypes, this idiotype did not increase significantly in titer during the first 30 d of the secondary immune response.

The HI titer of IdX detected by anti-B147 and SRBC-B109 was 3.5 ± 0.5 in nonimmunized animals. A slight decrease during the primary immune response was observed. However, the titer of the same idiotype increased promptly after secondary immunization and then decreased gradually.

To determine whether or not the IdX expressed early in the secondary response was attributable to antibody molecules with anti-HA specificity, we pooled the sera obtained 3 and 7 d after secondary stimulation and passed the pool through a B/Lee-Sepharose affinity column to separate the fractions that bound and did not bind to B/Lee virus. The two fractions then were tested in a conventional antiviral HI assay, and for IdX expression in HI assay using the anti-B147 and SRBC-B109 system. All antiviral activity was recovered in the fraction that bound to virus, but this fraction contained only 11% of the HI activity in the anti-B147 SRBC-B109 system. Conversely, the unbound fraction lacking detectable antiviral HI activity was responsible for 89% of the IdX expression. These results suggested that the majority of the IdX detected in the secondary response was due either to a parallel Ab₁ or to Ab₃. In an effort to distinguish between these two possibilities, we searched for the presence of Ab₂ (which could have provided the stimulus for Ab₃ synthesis) in sera obtained 20 and 30 d after primary immunization. Using direct hemagglutination assay with SRBC coated with B109, we were unable to detect anti-Id (i.e., Ab₂) antibodies in the late period of the primary immune response when IdX titers were declining.

Discussion

Although the structural basis for antigenic variation of influenza A virus HA molecules has been elucidated by recent sequence analysis of variants (8, 9), as yet little is known about the clonal nature of the anti-HA responses that confer immunity to infection. The availability of monoclonal antibodies to influenza virus HA and of techniques for raising anti-Id antibodies provide a potentially powerful tool for investigation of the idiotypes expressed during the immune response and for determining whether specific antibodies to antigenic variants are derived from a diverse set of clonotypes or from the progeny of common ancestor clones. In the present studies, on the basis of unchanged or altered reactivity of monoclonal antibodies with variants

selected in the presence of other monoclonal antibodies and detected frequencies of antigenic variants in the presence of pairs of monoclonal antibodies, P8, P20, and P28 were determined to be directed to different determinants on the PR8 virus HA, whereas the seven monoclonal antibodies to B/Lee virus were found to recognize overlapping determinants on the B/Lee virus HA (Tables II and III).

Analysis of the idiotypy of the three PR8 HA-specific monoclonal antibodies with homologous anti-Id antisera revealed extensive cross-reactivity, as assessed in HI assays and RIA (Table IV). This unusual idiotypic cross-reactivity has been found previously in other systems among antibodies to different determinants on the same molecule (22, 23), antibodies to different antigen molecules (24), and antibodies of unknown specificities (24, 25). The finding of shared IdX among P8, P20, and P28 monoclonal antibodies despite both their different antigenic specificities and the fact that they are of different IgG subclasses suggests that the IdX is encoded by a germ-line gene(s).

With respect to the mechanism responsible for shared IdX among monoclonal antibodies of different IgG subclasses specific for different HA determinants, several explanations can be entertained. (a) The clones associated with different antibody specificities but similar idiotype specificities are derived from one ancestral clone or V gene ancestor. During embryonic development and clonal expansion after antigen stimulation, somatic mutations may occur in V region genes so that different antibody specificities are generated, whereas idiotype specificities remain relatively unchanged (26). (b) The V_H region of immunoglobulin is encoded by separated V and J genes. During B cell maturation, V and J regions are juxtaposed, so it is possible that antibodies bearing different antigen binding specificities may have identical J segments (26, 27). (c) During B cell maturation, different minigenes (D genes) which code for 1-7 amino acids are inserted between V and J genes. This D gene may contribute to the distinct antigen-binding specificities, but antibodies may still have cross-reactive idiotypic determinants (23, 28). (d) The IdX represent(s) regulatory idiotype(s) capable of becoming dominant idiotypes because it is these determinants that stimulate idiotype-specific regulatory cells (29).

The data obtained in the present experiments do not permit us to distinguish among these possibilities. However, it should be noted that IdX were detected among PR8-specific and B/Lee-specific monoclonal antibodies, but no idiotypic cross-reactivity between the two groups of monoclonal antibodies was detected. These latter results suggest that the finding of IdX among monoclonal antibodies to different antigenic determinants on PR8 HA may reflect the effects of regulatory idiotopes (see *d*, above).

The study of the idiotypes expressed by monoclonal antibodies specific for B/Lee HA (Tables VI and VIII) demonstrated that these monoclonal antibodies bear three categories of idiotypic determinants: (a) idiotopes borne by a single monoclonal antibody, equivalent to an individual antigenic specificity of a myeloma protein (30), i.e., an IdI; (b) idiotypic determinants shared by a few but not all of the monoclonal antibodies; (c) IdX borne by monoclonal antibodies specific for the same or overlapping antigenic determinants on B/Lee HA.

It should be emphasized that the B/Lee-specific monoclonal antibodies used are of different subclasses (Table I). Moreover, although they are directed to overlapping antigenic determinants, the avidity with which individual monoclonal antibodies

bind to B/Lee has been found to vary over a wide range.² Hence, the IdX observed among these antibodies is not attributable to their being products of a particular clonotype. Nevertheless, the finding of IdX among antibody populations with overlapping but nonidentical specificities is not unexpected and could be explained by any of the four mechanisms discussed above.

In addition, it should be noted that with monoclonal antibodies to both PR8 and B/Lee binding to anti-Id antibody was inhibited by prior incubation of monoclonal antibodies with virus (Tables V and VIII). These results are compatible with the assumption that the idiotypic determinants are closely associated with the combining site. However, this does not mean that idiotypic determinants that no longer react with anti-Id after interaction with virus are necessarily combining-site associated. Indeed, Kunkel et al. (31) showed that binding of anti-Rh antibodies to a large antigen such as erythrocytes can lead to the disappearance of some idiotypic determinants and the appearance of other antigenic determinants that are different from those associated with the combining site.

Both persistence and variation of idiotypes during the immune response have been demonstrated in other systems. Thus, in humans it was shown that idiotypes of anti-Rh antibodies can persist for several years and in mice that the idio type of antigalactan antibodies can persist for 150 d (32–33). In contrast, it was shown in rabbits that some idiotypes of anti-*Salmonella typhi* (34), anti-*M. lysodeikticus* (35), or antiphenylarsonate (36), detected early after immunization, disappear and are replaced by new idiotypes in the late phases of the immune response. In the hen lysozyme system of mice, the IdX hen lysozyme was found only in the secondary immune response (37).

In general, the results we obtained from studies of the idiotypes expressed during immune responses of mice with B/Lee virus are in accord with these observations in that both persistence and variation of different idiotypes during primary and secondary immunization were observed. It should be noted that the IdI detected by anti-B147 and SRBC-B147 was not observable in the sera of mice after primary or secondary immunization with B/Lee virus. Some IdX were detectable in the sera of mice before immunization. The presence of IdX in nonimmunized mice could be explained either by antibodies with unknown specificities that could share the IdX of monoclonal antibodies or, alternatively, by the existence in the nonimmunized mice of a small amount of antibody to B/Lee HA, undetectable by antiviral HI assay but detectable by PFC assay (38).

The IdX detected by anti-B118 and SRBC-B123, and by anti-B118 and SRBC-B142, were expressed during both primary and secondary immune responses, indicating that such clones dominate the anti-B/Lee HA responses in adult BALB/c mice. In contrast, the IdX detected by inhibition of binding of anti-B142 to SRBC-B109 was observed only during the primary response. This absence of idio type during the secondary response could be explained by (a) the induction of an anti-Id antibody during the secondary response that clears the corresponding idio type from the blood, or (b) the appearance of suppressor T cells specific for the idio type that suppresses the expression of this idio type. These hypotheses are under investigation.

The level of idio type detected by anti-B147 and SRBC-B109 was high in nonimmunized mice, but was reduced after primary immunization. On the other hand, this idio type was expressed immediately after secondary stimulation. The high titers of

² Liu, Y.-N., and J. Schulman. Manuscript in preparation.

this idiotype during the secondary response could be explained by one (or more) of the following mechanisms: (a) expression during the secondary response but not the primary response of an Ab₁ (Id) having antigen specificity and bearing the IdX, (b) expression of a parallel Ab₁ bearing the IdX but lacking antigen specificity, (c) induction of Ab₃ (anti-anti-Id) that bears the IdX and either does or does not have antigen-binding specificity. Since the majority of IdX expressed early in the secondary response was found in the serum fraction that did not bind to virus, Ab₁ and Ab₃ with antigen specificity may be excluded as major sources of the IdX-bearing antibody seen in the secondary response. Although we could not detect anti-Id (Ab₂) antibody in sera obtained late in the primary response, we cannot exclude the possibility that Ab₂ antibody in concentrations too low to be detected with the assay system used, in the form of complexes, or sequestered in lymphoid organs, was present in quantities sufficient to induce an Ab₃ response. Hence, we are unable at present to distinguish whether the preponderance of IdX observed in the secondary response using the anti-B147 SRBC-B109 system is due to a parallel Ab₁ set or to Ab₃.

Based on this information on the variation of idiotypes during the anti-HA response, which reflects alterations in clonal expression, we intend to characterize the clones relevant for host defense mechanisms using either an idiotype suppression model or selective activation of clones by injection of minute amount of anti-Id antibodies at birth, which may lead to a B clonal dominance, as has been shown in the anti- β 2-6 fructosan A48Id system (39).

Summary

Anti-idiotype antisera were raised in syngeneic (BALB/c mice) and homologous (A/J mice) systems to study the cross-reactive idiotypes among monoclonal antibodies to PR8 and B/Lee virus HA and the expression of these idiotypes during primary and secondary antiviral responses of BALB/c mice. Extensive idiotypic cross-reactivity was demonstrated among monoclonal antibodies specific for distinct antigenic determinants on PR8 hemagglutinin (HA). The study of idiotype of monoclonal antibodies against the same or overlapping antigenic determinants on B/Lee HA showed that these monoclonal antibodies may bear (a) a true individual idiotype not shared by other monoclonal antibodies, (b) idiotypes shared by few monoclonal antibodies, and (c) true cross-reactive idiotypes shared by all of these monoclonal antibodies. In contrast, no cross-reactive idiotypes were detectable among monoclonal antibodies to B/Lee HA and monoclonal antibodies to PR8 HA. Furthermore, we have shown that the anti-idiotype antibodies we used recognize determinants on monoclonal antibodies closely associated with antigenic binding sites. Finally, studies of the idiotypes expressed during primary and secondary antiviral HA responses of mice immunized with B/Lee virus revealed persistence of some idiotypes during both primary and secondary responses, whereas others were only expressed in the primary or secondary response.

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