GENERATION OF ANTIBODY DIVERSITY IN THE IMMUNE RESPONSE OF BALB/c MICE TO INFLUENZA VIRUS HEMAGGLUTININ*

I. Significant Variation in Repertoire Expression between Individual Mice

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Much has been learned about the diversity of antibody responses to haptens and carbohydrates (1, 2), yet comparatively little is known about the generation of antibody diversity in the response to protein antigens. In the present study, we have analyzed the diversity of the adult BALB/c antibody response to the hemagglutinin (HA)[1] of the influenza A/PR/8/34 (PR8) virus using both paratypic and idiotypic analyses of anti-HA hybridoma antibodies.

The paratope of an anti-HA antibody can be defined by the antibody's fine specificity for a panel of antigenically related influenza A viruses. Previous studies have demonstrated extensive paratypic diversity in the BALB/c anti-HA response using a panel of eight epidemic influenza A viruses in the fine specificity analysis (3–5). More recently (6), we have refined the paratypic analysis by adding to the virus panel PR8 mutant viruses that possess single amino acid substitutions in the HA. In the present study we have used a panel of 39 distinct PR8 mutant viruses along with 12 epidemic influenza A viruses to characterize the fine specificities of 125 BALB/c anti-HA hybridoma antibodies. In general, anti-HA antibodies can be classified into four broad specificity groups, i.e., groups of antibodies having related fine specificities. We have suggested that these four specificity groups correspond to four antigenic sites (designated sites Sa, Sb, Ca, and Cb) on the HA (6). This is consistent with the four antigenic sites predicted from the recently published three-dimensional structure of the HA (7, 8). Thus, the paratope of an anti-HA antibody can be characterized in two related ways. First, an anti-HA antibody can be characterized by its specificity for one of the four antigenic sites on the HA. Second, antibodies binding to a given antigenic site can be further distinguished from each other on the basis of their patterns of reactivity with individual mutant viruses.

By combining this fine specificity analysis with an idiotypic analysis of anti-HA antibodies, we have addressed three questions: (*a*) what is the size of the anti-HA

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[1] *Abbreviations used in this paper:* BRB, Britton-Robinson buffer; DME, Dulbecco's modified Eagle's medium; DME-S, DME containing 7.5% fetal calf serum, 7.5% agamma horse serum, 2 mM glutamine, and 50 μg/ml gentamycin; H, heavy chain; HA, hemagglutinin; HAS, PBS, pH 7.2, containing 0.08% sodium azide; HAU, hemagglutinating units; Id, idiotype; IdX, cross-reactive Id; L, light chain; PBS, phosphate-buffered saline; PR8, influenza A/PR/8/34 virus; RIA, radioimmunoassay; RP, reactivity pattern; V, variable; V_H, H chain variable; V_κ, κ chain variable; V_L, L chain variable.

repertoire of adult BALB/c mice? (*b*) Is the anti-HA repertoire equivalently expressed in each individual BALB/c mouse? (*c*) What mechanisms are used to generate the diversity of the anti-HA repertoire and which germ line variable (V) genes are involved?

Materials and Methods

The Anti-HA Hybridoma Antibody Panel. Hybridomas were generated by fusion of lymphocytes from the spleen and/or mediastinal lymph nodes of 14 adult (8- to 16-wk-old) BALB/c mice after secondary or, in the case of mouse 35, primary immunization with influenza A/PR8/8/34 (PR8) (H1N1). The myelomas P3 × 63Ag8 (9), P3 × 63Ag8-cl.653 (10) or Sp2/0Ag14 (11) were used in these fusions that were performed as previously described (12). 125 of the hybridoma antibodies were shown to be reactive with the HA molecule of PR8 based on their reactivities with recombinant influenza viruses possessing known reassortments of genes of PR8 and the distantly related influenza virus A/Hong Kong/68 (H3N2) (13). Presently available evidence indicates further that these hybridoma antibodies are directed against antigenic determinants of the HA1 polypeptide (48,000 mol wt) of the HA molecule. The hybridomas were grown in vitro in Dulbecco's modified Eagles medium (DME) supplemented with 7.5% fetal calf serum and 7.5% agamma horse serum (both from Flow Laboratories, Rockville, MD), 2 mM glutamine, and 50 μg/ml gentamycin (DME-S). In the designation for each hybridoma, the first number after the letter H denotes the individual BALB/c mouse from which the hybridoma was generated.

Quantitation and Isotype Determination of Anti-HA Hybridomas. The concentration of anti-HA antibodies in culture fluids was determined in the viral radioimmunoassay (RIA) (see below) by comparing the binding of hybridoma antibodies to the binding of known quantities of affinity-purified BALB/c anti-PR8 antibody (14). Bound antibody was detected by means of radioiodinated rabbit anti-mouse-$(Fab')_2$ antibodies. The same viral RIA was also used to determine the heavy (H) chain isotype of the anti-viral antibodies. In the latter assay, bound hybridoma antibody was detected by incubation first with rabbit antisera specific for individual murine H chain isotypes and, second, with radioiodinated goat anti-rabbit antibodies (15).

Viruses. The PR8 mutant viruses were selected as described (16) by growing plaque-cloned parental virus PR8 [A/PR/8/34 (H1N1)], originally obtained from Mt. Sinai Hospital, New York, in the presence of individual anti-HA antibodies. Current evidence indicates that these viruses differ from the parental virus by single residue changes in the HA1 polypeptide (17). Each of the 39 PR8-mutants used in the present analysis exhibits an antigenically unique modification. In addition, 12 standard epidemic virus strains of the H1N1 subtype were used. The HA molecules of the latter virus strains differ from the HA molecule of PR8 by many residue changes yet the HA molecules remain serologically related. The presently used abbreviations and full designations of these viruses are: SW: A/Swine/31 (H1N1); WSN: A/WSN/33 (H1N1); MEL: A/Melbourne/35 (H1N1); BH: A/BH/35 (H1N1); HIC: A/Hickcox/40 (H1N1); BEL: A/Bellamy/42 (H1N1); WEI: A/Weiss/43 (H1N1); CAM: A/Cam/46 (H1N1); FM1: A/Fort Monmouth/1/47 (H1N1); FW: A/Fort Wyoming/1/50 (H1N1); MAL: A/Malaya/302/54 (H1N1); DEN: A/Denver/57 (H1N1).

All viruses were grown in embryonated hen's eggs, purified by banding in a sucrose gradient and quantitated by HA titration using chicken erythrocytes as previously described (14). The purified viruses were stored at 4°C in phosphate-buffered saline (PBS) pH 7.2, containing 0.08% sodium azide (HAS).

Viral RIA. Viral immunoadsorbents were prepared in two ways. In the case of PR8 mutant viruses, immunoadsorbent stock solutions were prepared by incubating 2×10^5 hemagglutinating units (HAU) of purified virus per ml HAS with 1% Nonidet P-40 for 30 min at 37°C. The virus solution was then diluted 10-fold with HAS and stored as a stock solution at 4°C. Immunoadsorbents were prepared by incubating 25 μl of a 1:100 dilution in HAS of the above stock solutions in individual wells of polyvinyl microtiter plates (Cooke Engineering, Alexandria, VA) overnight at room temperature. In the case of the epidemic virus strains, immunoadsorbents were prepared as described (12) by drying 25 μl of virus in HAS (corresponding to 20 HAU)

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into wells of polyvinyl mictotiter plates. Before use in the RIA, the plates were incubated for at least 1 h with HAS containing 1% bovine serum albumin.

The RIA was performed by incubating 25 μl of a dilution of hybridoma culture fluid (in HAS containing 10% agamma horse serum) for 90 min with the solid-phase viral immunoadsorbents. The wells were then washed, incubated for 90 min with 25 μl of radioiodinated rabbit anti-mouse-F(ab')$_2$ antibodies, washed, and the amount of radioactivity bound to individual wells determined in a gamma counter. Each culture fluid was first assayed to determine the concentration of antibody binding to the parental virus (PR8) immunoadsorbent. The fine specificity of each antibody was then determined as follows. A dilution of culture fluid containing 0.5–1 ng antibody/25 μl (referred to as the standard dilution) was tested for binding to the various viral immunoadsorbents. In parallel, serial $2^{-0.5}$ dilutions of the standard dilution were assayed on the parental virus to generate a standard binding curve for each antibody. The cpm observed in the interaction of the antibody with each heterologous virus was then related to the antibody's standard curve and was expressed, on a log 2 basis, as percent binding relative to the parental virus. The resulting reactivity pattern (RP) of each antibody represents the average of at least three independently performed assays. The final binding values have an average standard deviation of 0.37 (6).

Antibody Purification. All hybridoma antibodies were purified from serum-free hybridoma culture fluid (18). Hybridomas were first grown in DME-S. The cells were spun out of the medium (8 min, 300 *g*) when they had reached a density of ~10^6 cells/ml and were transferred to DME containing insulin (Sigma Chemical Co., St. Louis, Mo.) (5 μg/ml), transferrin (Sigma Chemical Co.), (5 μg/ml), glutamine (2 mM), and gentamycin (50 μg/ml). The culture fluid was collected every 24 h for 3 d by spinning the cells out of the medium and transferring them to fresh serum-free DME. The cell density was kept at 1.5–2 × 10^6 cells/ml.

Antibodies of γ2a, γ2b, and γ3 isotypes were purified by passing 500 ml of serum-free culture fluid (adjusted to pH 8) over a 7-ml Sepharose 4B-protein A column (Pharmacia Fine Chemicals, Piscataway, NJ). The antibody was eluted in a linear pH gradient (pH 7 to pH 3) using the 0.2 M Britton-Robinson buffer (BRB) consisting of 0.029 N citric acid monohydride, 0.058 N potassium phosphate monobasic, 0.029 N barbital, and 0.087 N boric acid. Hybridoma antibodies of γ1 isotype were concentrated from 500 ml of serum-free culture fluid by ultrafiltration using an immersible-CX ultrafilter (Millipore Corp., Bedford, MA), dialyzed against 0.2 M BRB pH 8 and then applied to the protein A column, which was equilibrated with 0.2 M BRB, pH 8. After washing the column with 15 ml of 0.2 M BRB, pH 8, the antibody was eluted using the same pH gradient as described above. Myeloma proteins were purified by standard methods from ascites. All purified antibodies were concentrated and dialyzed against PBS by ultrafiltration. Protein concentration was determined using the Biorad protein assay (Bio-Rad Laboratories, Richmond, CA).

Antiidiotypic Antisera. New Zealand white rabbits were injected subcutaneously in multiple sites with 300 μg of purified hybridoma antibody emulsified in complete adjuvant H37Ra (Difco Laboratories, Detroit, MI). The rabbits were boosted intramuscularly with 100 μg of purified hybridoma antibody in PBS, and sera were collected 10 d later. The antiserum prepared against H37-68 (γ1, κ) was absorbed with the myeloma protein MOPC-21 (γ1, κ) coupled to Sepharose 4B. The antiserum raised against H36-5 (γ3, κ) was absorbed with normal BALB/c immunoglobulin coupled to Sepharose 4B for use in the H36-5 idiotype (Id) assay to remove V_κ21 light (L) chain-specific antibodies contained in this serum. The anti-H36-5 antiserum used in the V_κ21C cross-reactive Id (IdX) assay was only absorbed with the myeloma protein J606 (γ3, κ). The rabbit antisera to κ chains from MOPC-70E (V_κ21A) and ABPC 22 (V_κ21B), raised as previously described (19–21), were kindly provided by M. Weigert and M. Julius (Institute for Cancer Research, Fox Chase, Philadelphia, PA), D. McKean (Department of Immunology, Mayo Medical School, Rochester, MN), and M. Potter (Laboratory of Cell Biology, National Cancer Institute).

Id Competition RIA. Absorbed anti-Id antisera were diluted (depending on antiserum, 1:10,000 to 1:25,000) in PBS, and 50 μl was added to wells of polyvinyl microtiter plates (Cooke Engineering, Alexandria, VA). The antibodies were allowed to adsorb to the wells by overnight incubation at room temperature. The plates were then incubated for at least 1 h with 1% bovine serum albumin in HAS before being used in the following competition assay: 25 μl of

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radioiodinated antibody (0.5–2 ng, corresponding to 10,000–20,000 cpm) and 25 μl of dilutions of unlabeled antibody test samples were added to each well. The diluent for labeled and unlabeled antibodies was HAS containing 10% agamma horse serum. The plates were then incubated for 3 h at room temperature, washed four times, and the radioactivity bound to individual wells was determined in a gamma counter. Each assay was standardized by using known quantities of unlabeled homologous antibody as competitor. In general, 1–4 ng of homologous antibody gave 50% inhibition of binding of the labeled antibody probe. Heterologous hybridoma culture fluids were screened at a 1:5 dilution. At this dilution, all culture fluids contained at least 5 ng (in general, >20 ng) of antibody per 25 μl. The purified hybridoma antibodies were radiolabeled with carrier-free Na ^{125}I (Amersham Corp, Arlington Heights, IL) using the chloramine-T method (22).

Production of Secondary BALB/c Anti-PR8 Sera. Adult BALB/c mice were immunized with 1,000 HAU of PR8 virus intraperitoneally. The mice were boosted 4 wk later with 1,000 HAU of PR8 intraperitoneally, and serum was collected individually after 10 d.

Results

The Size of the BALB/c Anti-HA Paratypic Repertoire. A total of 125 anti-HA hybridoma antibodies, generated from 14 individual BALB/c mice mostly after secondary immunization with PR8, were tested in the viral RIA for their binding to 39 PR8 mutant viruses and 12 standard epidemic virus strains of the H1N1 subtype. Table I shows that the antibodies exhibit 104 distinct reactivity patterns (RP). Consequently, the present panel of 125 anti-HA hybridoma antibodies contains at least 104 distinct clonotypes. Several small groups of antibodies exhibited identical RP. With three exceptions, the groups of antibodies having indistinguishable RP were derived from the same individual mouse. The antibodies within each of these groups may, but do not necessarily have to, possess identical combining sites. Based on the data in Table I, the most likely size of the adult BALB/c anti-HA repertoire was estimated to be 1,500 using the statistical methods of Wybrow and Berryman (23).

To investigate the generation of this large repertoire, we chose the following

TABLE I

Extent of Paratypic Diversity Among Anti-HA Hybridomas

Fusion number	Number of anti-HA hybridomas isolated	Number of distinct RP
1	1	1
2	8	8
3	2	2
8	7	7
9	4	4
16	2	1
17	5	4
18	11	8
20	1	1
28	5	5
33	3	3
35	9	9
36	13	11
37	54	43
Reactivity patterns repeated in two fusions		−3
Total	125	104

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approaches. First, given the apparent absence of a predominant anti-HA clonotype expressed at high frequency in each individual BALB/c mouse, we wanted to investigate further the relationships existing among anti-HA antibodies derived from the same individual mouse. For this reason, we saved all hybridomas obtained from the individual mice 36 and 37. Second, we screened the anti-HA hybridoma panel for the presence of Id that are markers for variable (V) region sequences derived from related germ line V genes. Given the previous demonstration that L chains belonging to the $V_\kappa 21$ group can be used in the anti-HA response of BALB/c mice (24, 25), we decided to concentrate on this group of germ line genes.

The Idiotypy and Fine Specificities of Antibodies Obtained from Individual Mice

THE ANTI-HA HYBRIDOMA PANEL FROM MOUSE 36. 13 anti-HA hybridomas were obtained from mouse 36 after secondary immunization with PR8, and the fine specificities of the hybridoma antibodies are shown schematically in Fig. 1. It is evident that this "individual" antibody panel is rather restricted in specificity in that it contains predominantly (10 out of 13) antibodies that bind to site Sb on the HA (i.e., nonreactivity with mutant viruses of the EV and/or BV mutant virus groups). In particular, many antibodies exhibit decreased binding to the mutant viruses BV1, BV12, and/or BV13. This RP has not been seen among 39 other antibodies of Sb specificity that were derived from other individual BALB/c mice.

A rabbit antiserum was raised against antibody H36-5. This antibody has been serologically shown to contain a L chain of the $V_\kappa 21C$ subgroup (see below). The antiserum was therefore absorbed with normal BALB/c serum immunoglobulin to remove anti-$V_\kappa 21C$ antibodies as well as anti-constant region antibodies. All 125 anti-HA hybridoma culture fluids were then tested for their capacity to inhibit the binding of radioiodinated H36-5 to this anti-Id antiserum. Only three culture fluids gave significant inhibition in this assay (>20%) and all three were from hybridomas (H36-5, H36-7, and H36-17) derived from individual mouse 36. When purified hybridoma antibodies from mouse 36 were assayed (Fig. 2), H36-15 also produced a significant inhibition of 30% when used at a concentration of 4.1 μg/25 μl. On the other hand, antibodies H36-1, H36-4, and H36-18 showed no inhibition even at high (4.1 μg/25 μl) concentration. This is particularly striking since H36-1 and H36-18 are indistinguishable in RP from the Id^+ antibodies H36-5 and H36-15, respectively.

When this same anti-Id serum was used with radiolabeled H36-17 in an Id competition RIA (Fig. 3), H36-5, H36-7, and H36-17 all gave 100% inhibition. Similarly, when radiolabeled H36-7 was used, all three antibodies gave 100% inhibition (data not shown). This implies that these three antibodies share some idiotypic determinants (i.e., the H36-5 Id). In these assays, H36-15 showed greater inhibition, but still only at high antibody concentrations. Thus, H36-15 must have idiotypic determinants that are weakly cross-reactive with some or all of the H36-5 Id determinants. On the basis of their shared idiotypic determinants and similar RP, H36-5, H36-7, H36-17, and H36-15 will be considered members of the H36-5 Id antibody family. Secondary anti-PR8 antisera from 75 individual BALB/c mice, containing on average ~200 μg/ml of anti-HA antibody, were screened for the presence of the H36-5 Id using this assay. No antiserum gave >30% inhibition at a 1:20 dilution. Therefore, the concentration of H36-5 Id within these antisera must be <0.4 μg/ml. Thus, the

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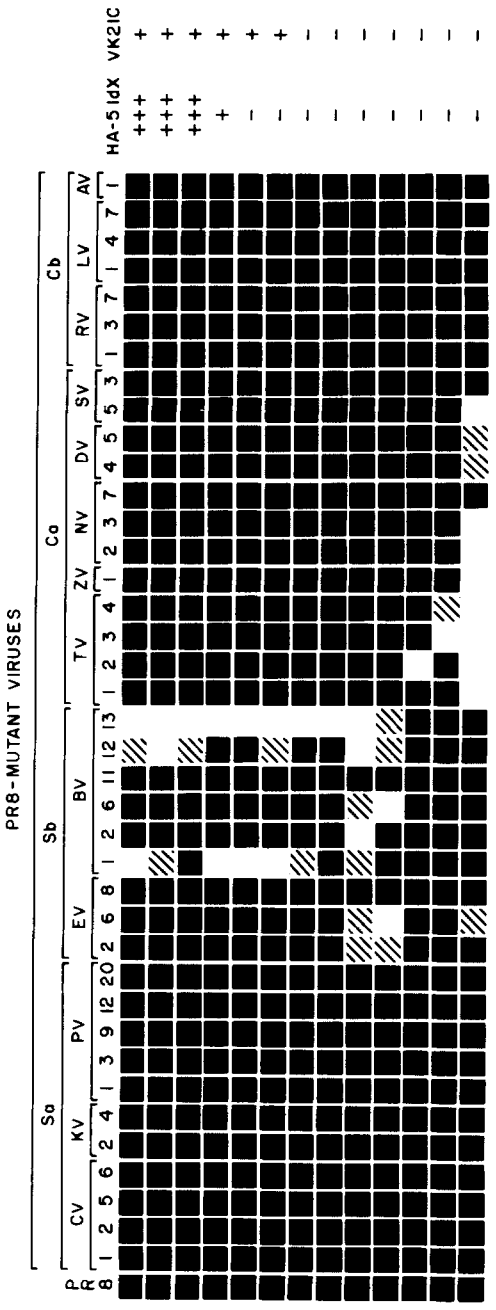

FIG. 1. RP analysis of anti-HA antibodies obtained from individual mouse 36 on PR8 mutant viruses. Each square represents percent binding to the PR8 mutant virus relative to binding to PR8 expressed on a $\log_2$ basis (6.64 = 100%) as described in Materials and Methods. ■: Full binding (5.64–6.64); □: moderately decreased binding (4.64–5.64); ▨: low binding (<4.64). See text for details of the Id analysis.

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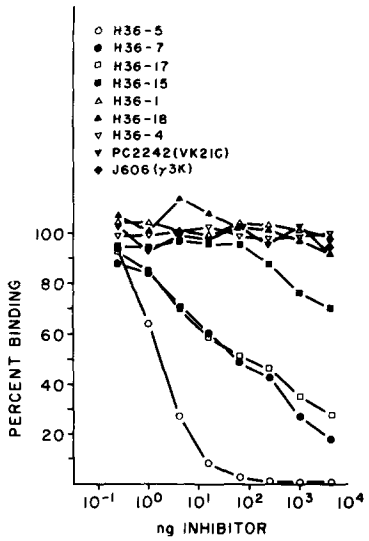

FIG. 2. Id competition RIA using rabbit anti-H36-5 with ^{125}I-labeled H36-5. The percent binding (average of triplicate samples) of the iodinated antibody was determined according to: [(cpm in presence of competitor) − BKG/(cpm in absence of competitor) − BKG] × 100. The background (BKG) is the cpm in the absence of rabbit anti-H36-5.

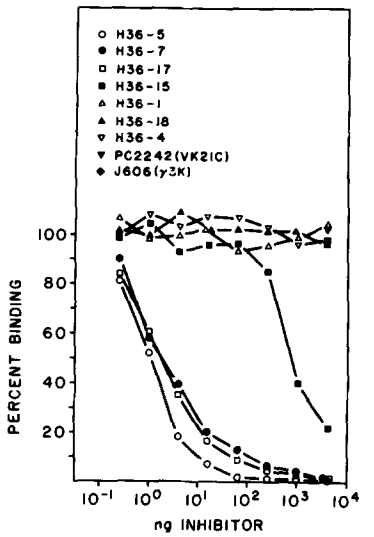

FIG. 3. Id competition RIA using rabbit anti-H36-5 with ^{125}I-labeled H36-17. See legend to Fig. 2.

H36-5 Id is a rare anti-HA Id that is nevertheless expressed on 31% of the hybridoma antibodies isolated from mouse 36.

THE ANTI-HA HYBRIDOMA PANEL FROM MOUSE 37. A panel of 54 anti-HA hybridomas was isolated from the individual adult BALB/c mouse 37. The antibodies from these hybridomas were found to have 43 distinct RP when tested on the panel of PR8 mutant viruses (Fig. 4) and on the 12 epidemic influenza A viruses (data not shown).

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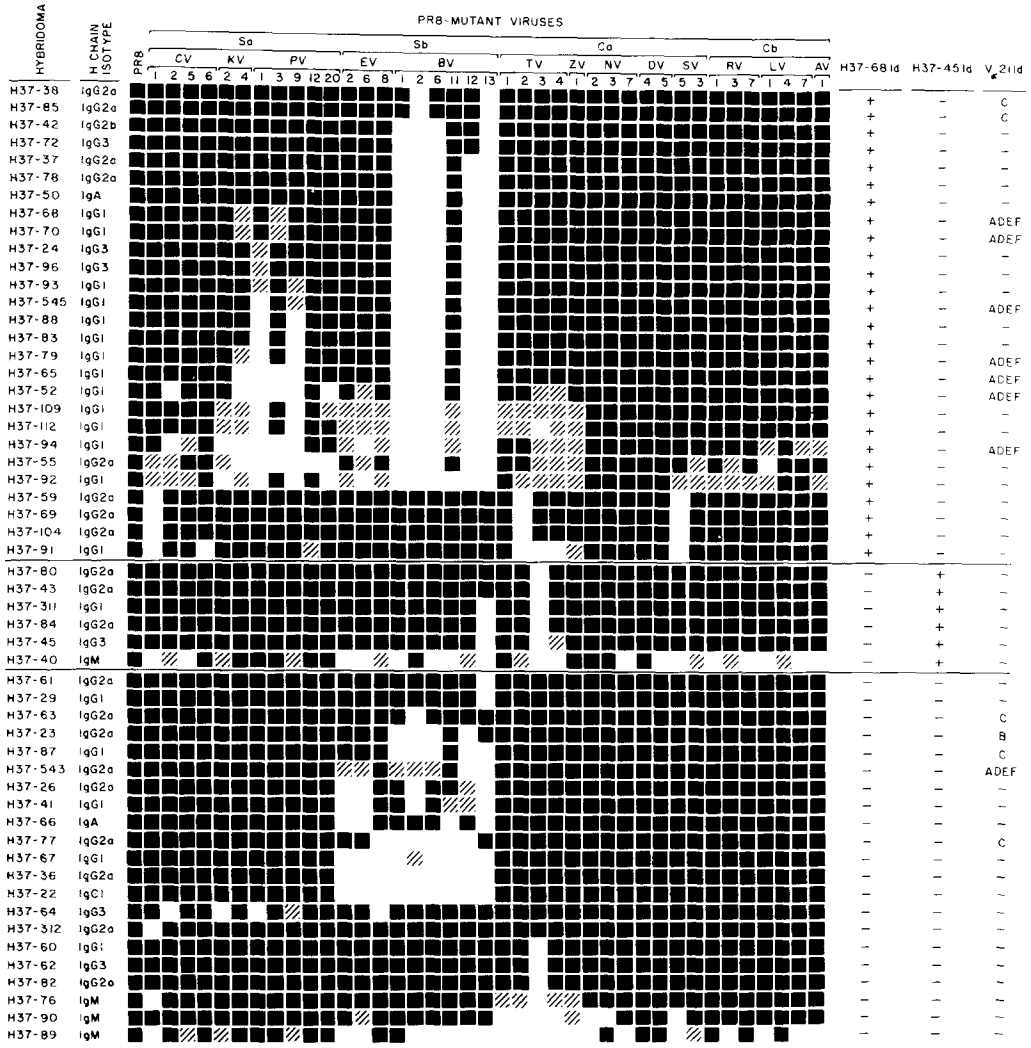

FIG. 4. RP analysis of anti-HA antibodies obtained from individual BALB/c mouse 37 on PR8 mutant viruses. See legend to Fig. 1. See text for details of the Id analysis. The H37-45 Id will be described in a separate publication (manuscript in preparation).

Two of these RP have also been observed among antibodies isolated from other individual BALB/c, but the majority of the RP are unique to mouse 37. Furthermore, the fine specificity analysis revealed large families of paratypically similar but not identical antibodies. For example, many antibodies showed decreased binding to BV1, BV2, BV6, BV12, and BV13 with and without decreased binding to other mutant viruses. Similarly, a number of antibodies showed decreased binding to BV13 and/or TV3. Thus, there are clear paratypic similarities among the antibodies from mouse 37, suggesting that groups of these antibodies have structurally related V domains.

To further define the structural relationships between these antibodies, a rabbit antiserum was raised against hybridoma antibody H37-68. This antiserum was

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absorbed with appropriate myeloma proteins to remove anti-constant region specificities and was then used to set up an Id competition RIA with H37-68 as the radiolabeled probe. This assay was fully inhibitable by purified PC6308, a myeloma protein containing a V_κ21D L chain. Furthermore, when culture fluids from the 125 anti-HA hybridomas were screened in this assay, the 10 culture fluids that gave full inhibition were the same ones that fully inhibited the V_κ21ADEF assay (see below). Therefore, this assay most likely detected V_κ21 L chain Id determinants.

To circumvent this problem, the rabbit that had been immunized twice with H37-68 was boosted with H37-24, a V_κ21ADEF IdX^- antibody (see Fig. 4) that is paratypically similar but not identical to H37-68. After absorption with myeloma proteins to remove anti-constant region specificities, this antiserum was used to set up an Id competition RIA using radiolabeled H37-24. This assay, termed the H37-68 Id assay, was not inhibited by the V_κ21D myeloma protein PC6308. When the anti-HA hybridoma culture fluids were assayed for the H37-68 Id, 28 culture fluids gave between 50 and 100% inhibition, while the remaining 97 culture fluids gave between 0 and 30% inhibition. 27 of the H37-68 Id^+ antibodies were derived from mouse 37 and thus represent 50% of all anti-HA hybridoma antibodies isolated from that mouse. One H37-68 Id^+ antibody was isolated from mouse 18. Therefore, only 1.4% of the hybridoma antibodies isolated from 13 BALB/c mice other than mouse 37 were positive for the H37-68 Id.

When purified hybridoma antibodies were used as inhibitors in the H37-68 Id assay, it became clear that the H37-68 Id is heterogeneous (Fig. 5). Four of the purified antibodies tested had identical inhibition curves and gave full inhibition, but three antibodies only gave 60–70% inhibition at high antibody concentration. Although these results demonstrate the heterogeneity of the H37-68 Id, it is clear that

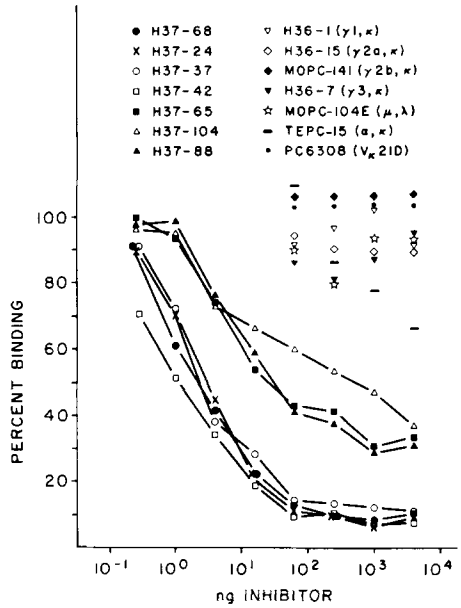

FIG. 5. Id competition RIA using rabbit anti-H37-68 with ^{125}I-labeled H37-24. See legend to Fig. 2.

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there must be extensive sharing of idiotypes among the H37-68 Id^+ antibodies, since they all gave >50% inhibition in the H37-68 Id assay.

The RP of the 27 H37-68 Id^+ antibodies from mouse 37 (Fig. 4) illustrate two important points. As noted above, a large group of paratypically related antibodies, characterized by reduced binding to BV1, BV2, BV6, BV12, and BV13, was isolated from mouse 37. These antibodies, with the exception of H37-87 and H37-543, were all H37-68 Id^+, which strengthens the possibility that these antibodies have structurally related V domains. On the other hand, there is considerable paratypic diversity among these H37-68 Id^+ antibodies, in that the antibodies exhibited 18 distinct RP in the fine specificity analysis. Furthermore, four of these antibodies (H37-59, H37-69, H37-104, and H37-91) appear to be paratypically quite dissimilar from the rest of the H37-68 Id^+ antibodies. In summary, the H37-68 Id defines a family of antibodies that (*a*) is disproportionately represented among the antibodies isolated from mouse 37, (*b*) displays idiotypic microheterogeneity, and (*c*) consists of both paratypically similar and dissimilar antibodies.

Anti-HA Antibodies Bearing $V_\kappa 21$ Group-related L Chain Id. To identify germ line V genes that are used to generate the diverse anti-HA repertoire, we made use of Id that are found on κ chain variable (V_κ) sequences belonging to the same V_κ group. V groups consist of V sequences that are highly homologous within the first framework region (2). Within each V group, subgroups are defined by a linked series of subgroup specific residues that are scattered throughout the V region in both framework and complementary determining regions. In the best-studied V_κ group, $V_\kappa 21$, six subgroups (A–F) have been defined (19, 20). Members of a $V_\kappa 21$ subgroup are thought to be derived from a single germ line V gene with or without somatic mutation (19, 20, 26). Thus, idiotypic determinants formed by subgroup-specific residues should be ideal markers for a given germ line V gene (19–21).

Three different competition RIA for $V_\kappa 21$ subgroups were set up to screen the anti-HA hybridoma panel for the presence of these L chains. The assay shown in Fig. 6 a was most sensitive for $V_\kappa 21C$, but also detected $V_\kappa 21D$, though to a lesser extent. A second assay (Fig. 6 b) detected predominantly $V_\kappa 21B$, but $V_\kappa 21C$ also gave partial inhibition. The third assay (Fig. 6 C) used an antiserum that was characterized previously (21) as reacting most strongly with the $V_\kappa 21A$, $V_\kappa 21D$, $V_\kappa 21E$, and $V_\kappa 21F$ subgroups and partially with the $V_\kappa 21C$ and $V_\kappa 21B$ subgroups.

The anti-HA hybridoma culture fluids were tested for inhibition in these three assays at dilutions containing between 10 and 100 ng of antibody. A hybridoma culture fluid was scored as $V_\kappa 21C$ IdX^+ if it gave >80% inhibition in the $V_\kappa 21C$ assay and <50% inhibition in the $V_\kappa 21B$ and $V_\kappa 21ADEF$ assays. Analogous criteria were used to score hybridoma culture fluids as either $V_\kappa 21B$ IdX^+ or $V_\kappa 21ADEF$ IdX^+. Of the 125 anti-HA hybridoma antibodies tested, 16 were positive for $V_\kappa 21C$ IdX, 1 for $V_\kappa 21B$ IdX, and 10 for $V_\kappa 21ADEF$ IdX. Thus, 22% of the anti-HA hybridomas use $V_\kappa 21$ L chains. The $V_\kappa 21$ IdX^+ antibodies were isolated from seven individual BALB/c mice.

The RP of the $V_\kappa 21$ IdX^+ hybridoma antibodies in the PR8 mutant virus analysis are shown in Fig. 7. 23 of the 27 antibodies bind to epitopes in or near the Sb antigenic site since they have low reactivity with viruses in the Sb mutant group (i.e., EV and BV mutants). Of these 23 antibodies, the $V_\kappa 21C$ IdX^+ and $V_\kappa 21B$ IdX^+ antibodies are clearly specific for site Sb. On the other hand, most of the $V_\kappa 21ADEF$

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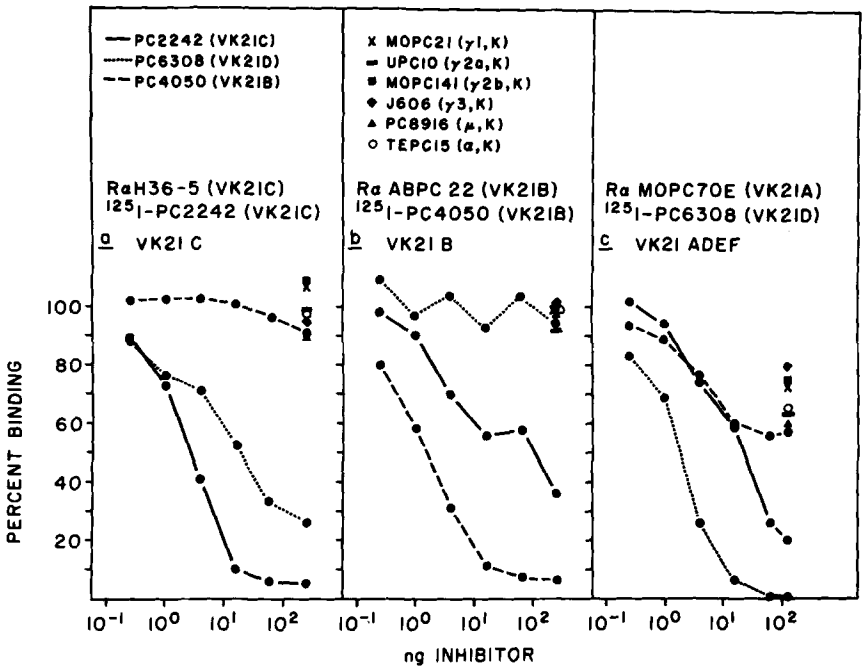

FIG. 6. $V_\kappa 21$ IdX competition RIA. See legend to Fig. 2.

IdX^+ antibodies also have decreased binding to some viruses in the Sa mutant group, suggesting that these antibodies bind near the junction of sites Sa and Sb. Of the remaining four $V_\kappa 21$ IdX^+ antibodies, two are directed against site Cb and two against site Ca. Thus, the $V_\kappa 21$ IdX^+ antibodies bind predominantly, but not exclusively, to site Sb on the HA.

Discussion

The present analysis of 125 anti-HA hybridoma antibodies provides unequivocal evidence of the large diversity of the adult BALB/c anti-HA antibody response. Thus, on the basis of the reactivity of these antibodies with 51 antigenically related HA molecules, we could differentiate 104 paratypically unique antibodies. Paratypically indistinguishable antibodies obtained from different donor mice were observed in only three instances. Based on this repeat frequency, the most likely size of the adult BALB/c anti-HA paratypic repertoire is 1,500, as calculated by the method of Wybrow and Berryman (23). This estimate exceeds several-fold a previous estimate of the paratypic repertoire size based on a similar analysis of monoclonal anti-HA antibodies produced in the splenic fragment culture system (3–5). The latter studies were performed, however, with a considerably smaller panel of cross-reative HA antigens and, as a consequence, failed to dissect two major specificity groups (i.e., Sa and Sb) of the BALB/c anti-HA repertoire into their individual paratypic components. It is very likely that the present estimate is still less than the actual size of the anti-HA repertoire of adult BALB/c mice. First, the inability to differentiate two antibodies from each other by the present mutant virus analysis does not prove their paratypic identity. Second, one of the assumptions made in calculating the repertoire

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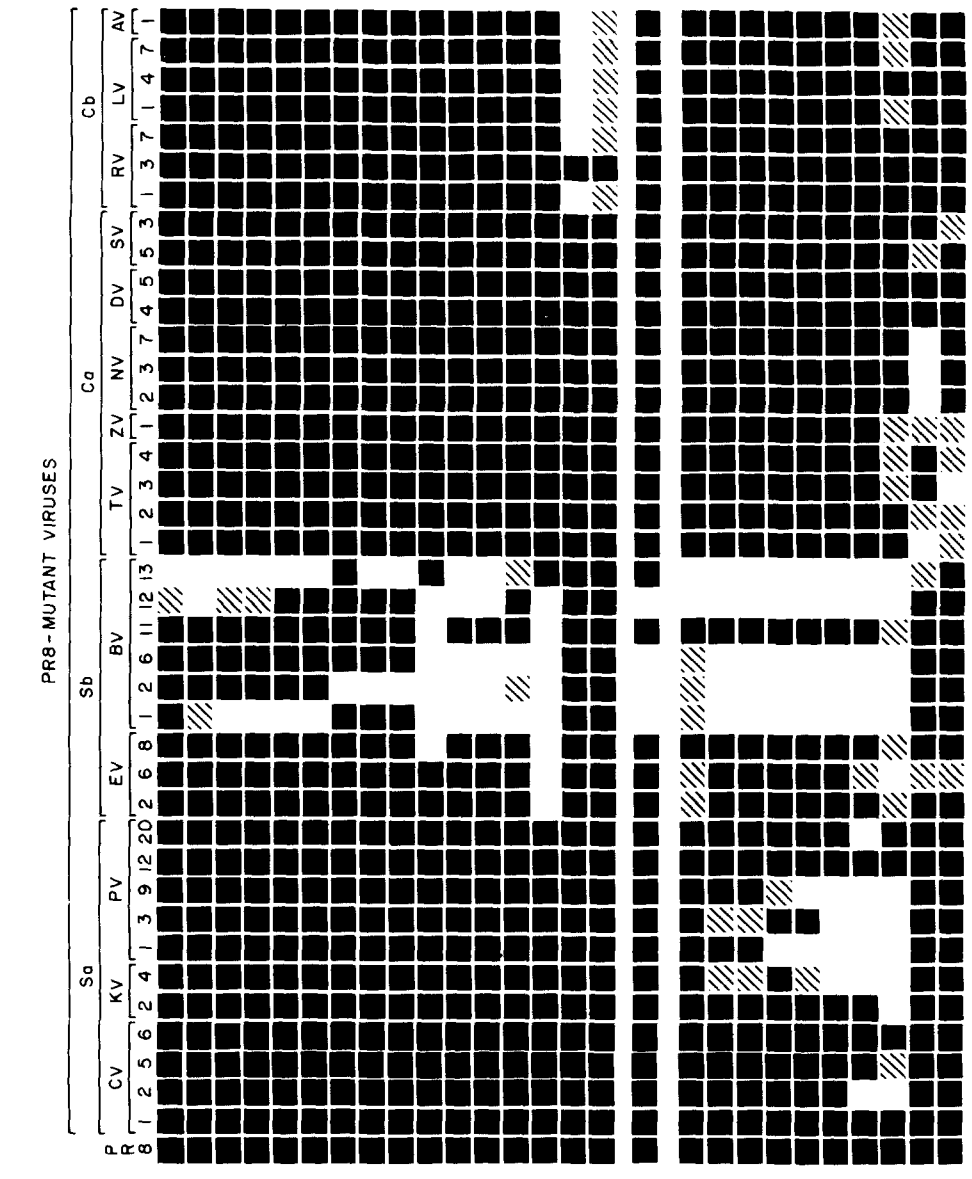

FIG. 7. RP analysis of V$_\kappa$21 Id$^+$ antibodies on PR8 mutant viruses. See legend to Fig. 1.

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size is that each clonotype has an equal chance of selection in each individual mouse. However, the analysis of anti-HA antibodies from individual BALB/c mice in the present report suggests that certain Id can be present at a high frequency among antibodies from one individual yet be missing entirely among antibodies isolated from another individual. Finally, it should also be noted that the present estimate measures only that part of anti-HA sequence diversity which affects the paratypic properties of an antibody, and thus probably represents only a fraction of the total BALB/c anti-HA diversity.

Despite the extensive diversity of the anti-HA repertoire, there are families of anti-HA antibodies that have structurally related V regions. Thus, the $V_\kappa 21$ idiotypic analysis defined a family of 27 anti-HA antibodies derived from seven individual BALB/c mice that bear L chains belonging to the $V_\kappa 21$ group. This family constitutes 22% of our anti-HA hybridoma panel. Previous studies have shown that ~8.6% of normal BALB/c κ-bearing serum immunoglobulin is $V_\kappa 21$ IdX^+ (21). Assuming that our anti-HA hybridoma panel is a fair cross-section of the BALB/c anti-HA repertoire, these findings suggest that the $V_\kappa 21$ group is used preferentially in the anti-HA response. In other words, out of an estimated 300 germline V_κ genes (19, 20, 27–29) only 6–8 related V_κ genes coding for the $V_\kappa 21$ group (19, 20, 26) are used to generate more than one-fifth of the anti-HA repertoire.

Additional families of idiotypically related anti-HA antibodies were observed among antibodies isolated from the same individual BALB/c mouse. Among the 13 anti-HA antibodies isolated from mouse 36, 4 shared the H36-5 Id, which was not detected among 112 anti-HA antibodies derived from 13 other individual BALB/c mice or in secondary anti-PR8 antisera from 75 individual BALB/c mice. The RP of these four H36-5 Id^+ antibodies on the panel of PR8 mutant viruses are quite similar to each other but clearly different from the RP of the anti-HA antibodies that were not derived from mouse 36. Why was the H36-5 Id only found among anti-HA antibodies from one individual mouse? We suggest that the H36-5 Id^+ antibodies were generated by a process of somatic diversification that took place in mouse 36 and is statistically unlikely to occur in exactly the same fasion in other individual BALB/c mice. We further suggest that the B cells producing H36-5 Id^+ antibodies descended from a common precursor cell in mouse 36 and that the paratypic and idiotypic diversity among these antibodies is due to somatic mutation (Fig. 8).

To verify that the H36-5 Id family descended from a common precursor cell by somatic mutation, it must first be shown that the members of the H36-5 Id family are using somatic V genes that are generated from the same germ line H chain variable (V_H) and L chain variable (V_L) genes. The $V_\kappa 21$ idiotypic analysis demonstrated that all members of the H36-5 Id family use $V_\kappa 21C$ L chains and thus probably have somatically generated V_κ genes derived from the same germ line V_κ gene (19, 20, 26). Interestingly, antibodies H36-1 and H36-18, which lack the H36-5 Id, also use $V_\kappa 21C$ L chains and are paratypically quite similar to the H36-5 Id family. This suggests that the latter two antibodies are also clonally related to the H36-5 Id family but have lost the H36-5 Id in the course of somatic diversification. This possibility is supported by a DNA restriction enzyme analysis of the $V_\kappa 21C$ IdX^+ hybridomas from mouse 36, which indicated that these hybridomas all have productively rearranged the same V_H and V_L genes (R. Luedtke and M. Weigert, manuscript in preparation).

Additional evidence for the common clonal origin of the $V_\kappa 21C$ IdX^+ cells from

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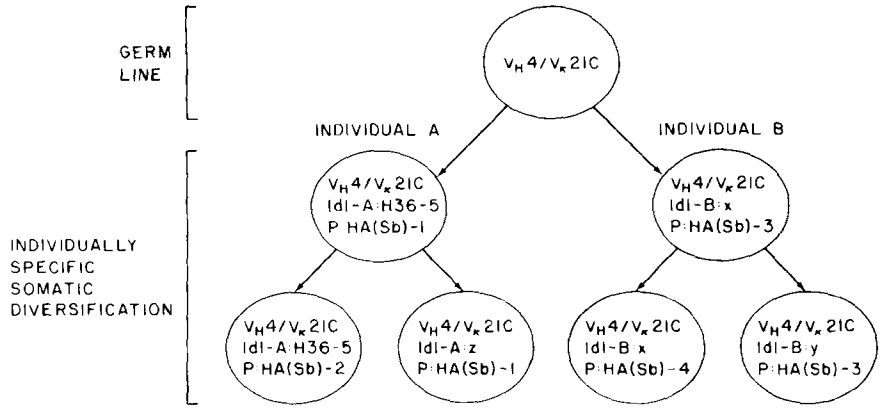

FIG. 8. Somatic diversification may generate differences in anti-HA repertoire between individual BALB/c mice. This schematic is based on the family of V_κ21C IdX^+ antibodies isolated from mouse 36 (see text for details). The model proposes that from a given precursor B cell with rearranged V_H and V_L germ line genes in individual A, a clonotype could be somatically generated that has an individual Id (IdI-A:H36-5) that is a marker for the particular process of somatic diversification that took place in that individual mouse. The paratope (P) of this clonotype is specific for site Sb on the HA and has fine specificity 1 [HA(Sb)-1]. Further somatic diversification can vary the IdI and the P independently. Starting with the same precursor B cell, individual B is unlikely to somatically generate a clonotype with the same IdI as was generated in individual A.

mouse 36 comes from sequence studies of the somatically generated V domains. Amino terminal V_H sequences from this family belong to the V_H4 group and are very similar to the translated protein sequence of gene V11, one of the TEPC15-related germline V_H genes recently sequenced by Crews et al. (30) (R. Luedtke, D. McKean and M. Weigert, manuscript in preparation). Furthermore, the amino terminal V_L sequences of these antibodies are, as predicted, closely related to the V_κ21C germline sequence (19). (D. McKean, R. Luedtke, and M. Weigert, manuscript in preparation). However, each L chain differs by several amino acids from the germline sequence. Notably, a number of these substitutions are shared by three to five of the antibodies within this family. These findings strongly support the notion that at least subsets of this family were derived from a common progenitor B cell possessing particular somatic mutations that were conserved in its progeny. Each antibody within this family is a distinct clonotype, as judged by the idiotypic and paratypic analyses. Therefore, if all the V_κ21C IdX^+ cells from mouse 36 did, in fact, have a common clonal origin, then the somatic mutational process was apparently quite active and may reflect the action of a specialized hypermutational mechanism such as that postulated by Kim et al. (31).

The H37-68 Id, like the H36-5 Id, defined a family of antibodies that was predominantly expressed in a single individual mouse. However, a major difference between H37-68 Id family and the H36-5 Id family is that the H37-68 Id^+ antibodies appear to have L chains of at least three different V_κ subgroups based on the V_κ21 IdX assays (see Fig. 4). These assays detected 2 H37-68 Id^+ antibodies that have L chains of the V_κ21C subgroup, 7 antibodies that have L chains of the V_κ21A, D, E, or F subgroups, and 18 antibodies whose L chains are serologically unrelated to any known V_κ21 L chain subgroup. Since the serological assays used here are able to detect L chains of all known V_κ21 subgroups, and since each V_κ subgroup is most

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likely derived from a separate germ line gene (19, 20, 26), one would have to conclude that the H37-68 Id^+ antibodies possess L chains derived from at least three different germ line V_κ genes. Consequently, these data seem to imply that the H37-68 Id family did not descend from a common precursor cell (containing both productively rearranged V_H and V_L genes) as hypothesized for the H36-5 Id family (above). However, if the $V_\kappa 21$ idiotypic determinants were altered in the course of somatic diversification, either by somatic mutation, somatic recombination between related V genes (28, 32), or gene conversion (33), then the H37-68 Id family could have had a common clonal origin. Alternatively, the H37-68 Id family could have descended from a common pre-B cell with a productively rearranged V_H gene (34, 35) which subsequently rearranged various V_L genes. Ultimately, structural analysis of the V domains within the H37-68 Id family should elucidate the clonal history of this family.

Both the H36-5 Id and the H37-68 Id, although rarely expressed within the BALB/c strain as a whole, were found on large and diverse families of hybridomas isolated from single individual mice. Recently, other investigators (36) demonstrated that three anti-HA (PR8) hybridoma antibodies had distinct paratypes and yet all shared an Id. Interestingly, these hybridomas constituted all of the anti-HA (PR8) hybridomas obtained from one individual BALB/c mouse (J. Schulman, personal communication). Although anti-HA (PR8) hybridomas from other individuals were not assayed for this Id, these findings are consistent with the present observation of idiotypic sharing by diverse anti-HA antibodies isolated from the same individual mouse.

What factors could account for the dominance of rare Id within the expressed repertoires of individual mice? First, suppose that the B cells bearing such a rare Id have a polyclonal origin within an individual mouse. In this case, the regulatory mechanisms leading to the dominance of these clonotypes must be directly or indirectly related to the Id. For example, idiotypic network interactions (37) may differ among individuals leading to the expansion of rare, idiotypically related clonotypes within a given individual mouse. Also, individual differences in antigenic history might exist such that a particular individual may have been exposed to a unique set of environmental antigens that primed B cells cross-reacting with the HA. These clonotypes might possess a common Id and could, upon subsequent experimental immunization with the HA, dominate the individual's anti-HA response. Alternatively, suppose that the B cells bearing a rare Id within an individual mouse are all derived from a single precursor B cell by somatic mutation, as discussed above. The progeny of a single precursor B cell might well share a rare Id and thus the regulatory mechanisms mentioned above may, but do not necessarily have to contribute to the expansion of these clonotypes within an individual. Conceivably, the somatic mutational process within the clonal progeny of a precursor B cell might be coupled with differentiation of these B cells into rapidly proliferating cells that could eventually dominate an individuals's anti-HA repertoire.

Summary

The paratypic and idiotypic diversity of the BALB/c antibody response to the hemagglutinin (HA) of the influenza A/PR/8/34 virus (PR8) was investigated using a panel of 125 anti-HA hybridoma antibodies derived from 14 BALB/c mice. The paratypic diversity, as assessed by a fine specificity analysis using 51 related influenza

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viruses, was extensive: 104 distinct paratopes were observed. In three instances, antibodies with indistinguishable paratopes were isolated from two individual mice. A minimum estimate of the size of the adult BALB/c anti-HA paratypic repertoire, calculated from these data, is 1,500.

The generation of this diverse repertoire was studied by screening the anti-HA hybridoma panel for the presence of idiotypes (Id) that are markers for variable (V) region sequences derived from related germ line V genes. Three cross-reactive Id (IdX) that are markers for the V_κ21C, V_κ21B, and V_κ21A, D, E, or F L chain subgroups were found, respectively on 16, 1, and 10 anti-HA hybridoma antibodies derived from seven individual BALB/c mice. Thus, the V_κ21 IdX^+ hybridomas constitute 22% of the anti-HA hybridoma panel. The V_κ21 IdX are also present on 8.6% of κ-bearing immunoglobulin in normal BALB/c serum. This suggests that the V_κ21 group is used preferentially in the BALB/c anti-HA immune response.

The generation of the anti-HA repertoire was further studied using large panels of anti-HA hybridomas derived from two individual adult BALB/c mice. Anti-idiotypic antisera were raised in rabbits against individual hybridomas from each mouse. One anti-Id serum defined a family of four idiotypically and paratypically related, but not identical, antibodies from mouse 36, which represented 31% of the hybridoma antibodies isolated from this mouse. None of the 112 anti-HA hybridoma antibodies derived from 13 other individual mice showed idiotypic cross-reactivity. Furthermore, this Id could not be detected in anti-PR8 antisera from 75 individual BALB/c mice. Another anti-Id serum defined a family of 27 idiotypically related antibodies from mouse 37, which represented 50% of the hybridoma antibodies isolated from this mouse. Only 1 of the 71 hybridoma antibodies isolated from 13 other individuals was idiotypically cross-reactive.

These results demonstrate that individual adult BALB/c mice express paratypically and idiotypically distinct antibody repertoires to the HA of influenza virus PR8. Based on these observations, we suggest that somatic mutation plays an important role in the generation of the adult anti-HA repertoire. Mechanisms that could account for differences in repertoire expression among individual mice are discussed.

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