JOURNAL OF VIROLOGY, June 1989, p. 2758–2767
0022-538X/89/062758-10$02.00/0

Vol. 63, No. 6

Induction of Immune Response to Influenza Virus with Anti-Idiotypic Antibodies

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Received 5 December 1988/Accepted 7 March 1989

Anti-idiotypic (anti-Id) antibodies were raised in rabbits against five monoclonal antibodies (MAbs) specific for different antigenic sites on the hemagglutinin (HA) of influenza virus $Mem71_H$-Bel_N (H3N1) [A/Memphis/1/71 (H3N2) × A/Bel/42 (H1N1)]. Each of the anti-Id sera was directed predominantly towards a unique (private) idiotype of the immunizing MAb, none of the five idiotypes being detectable in pooled BALB/c antisera against $Mem71_H$-Bel_N virus or on most other anti-HA MAbs tested. Partial idiotypic sharing was observed, however, between certain MAbs, from different mice, having the same or similar epitope specificity for HA. When used as immunogens in BALB/c mice, two of the five anti-Id preparations induced antibodies that reacted with $Mem71_H$-Bel_N virus and displayed neutralizing activity. Mice of other inbred strains responded similarly, indicating that the response was not genetically restricted by the *Igh* locus. From their pattern of reactivity with mutants of $Mem71_H$-Bel_N virus with known single amino acid substitutions in the HA molecule, the antiviral antibodies elicited by anti-Id antibodies were shown to be directed to the same antigenic site on A/Memphis/1/71 HA as the original immunizing MAb (site A or site E, respectively). However, several of these antisera were shown to contain additional distinct subpopulations of antibodies specific for heterologous influenza A virus strains, either of the H3 subtype or of a different HA subtype (H1 or H2). Since the induction of antibodies to HA of different subtypes is not a feature of the antibody response to influenza virus itself, their induction by anti-Id antibodies merits further investigation.

The antigenic determinants that are associated with the variable region of an antibody molecule constitute its idiotype (Id). Anti-idiotypic (anti-Id) antibodies (Ab2) raised against the Id of Ab1 may be of two types (17). Ab2β bear idiotypic structures that are complementary to the paratope of Ab1 and represent an "internal image" of the antigenic epitope recognized by Ab1. In contrast, Ab2α bind through their paratopes to idiotopes associated with the framework or paratope of Ab1 and bear no structural resemblance to antigen. Studies in a variety of model systems have shown that it is possible to induce specific antibody and/or T-cell responses to a given antigen by immunizing with anti-Id antibodies (for example, see references 2, 5, 9, 10, 18, 29, 31, 33, and 36). Both types of anti-Id antibody could theoretically induce such responses: Ab2β, because of its structural homology with antigen, and Ab2α, if the idiotope against which it is directed recurs on immunoglobulin molecules (or T-cell receptors) of a particular antigen specificity.

Influenza virus represents an interesting model for the study of anti-Id antibodies as immunogens. Protective immunity to influenza virus is largely strain specific and is mediated by antibody directed against the hemagglutinin (HA) molecule, one of the best characterized of all viral antigens. The crystal structure of HA of the H3 subtype is known (41), as are the amino acid sequences of the HAs of numerous influenza virus strains, including both natural variants arising by antigenic drift (3, 37) and neutralization-resistant "escape" mutants selected in the laboratory by growth in the presence of individual anti-HA monoclonal antibodies (MAbs) (11, 20, 38). The antibody response to HA is extremely diverse, and for H3 HA, the epitopes recognized by antibodies have been shown to cluster into five major antigenic sites on the globular head of the HA molecule (Fig. 1) (3, 39, 40), although considerable overlap between sites exists. Previous idiotypic analyses of anti-HA MAbs revealed the presence both of unique (private) Ids and of cross-reactive Ids which were shared by different individuals of the species and present on antibodies of different epitope specificity (21, 24, 35). HA is also a major influenza antigen recognized by helper T (T_h) cells, and in recent years the locations of a number of epitopes recognized by T_h cells from different inbred strains of mice and from humans have been identified to the level of short antigenic peptides (14, 15, 19, 23).

We have commenced a study of the nature and fine specificity of the immune response to influenza virus induced by anti-Id antibodies, comparing it with that induced by the virus itself. In this report we describe the production and characterization of rabbit anti-Id antibodies against five anti-HA MAbs specific for distinct or overlapping antigenic sites on the HA of influenza virus A/Memphis/1/71 (Mem71) (H3 subtype). We show that neutralizing antibody specific for influenza virus can be induced in mice by immunization with anti-Id antibodies, and we examine the fine specificity of the antiviral antibodies induced by two different anti-Id preparations.

MATERIALS AND METHODS

Mice. BALB/c, CBA, C57BL/10, and B10.D2 mice were bred in the animal facility of our department. Female mice were used between 6 and 10 weeks of age.

Viruses. The type A influenza viruses used were $Mem71_H$-Bel_N (H3N1), a genetic reassortant of A/Memphis/1/71 (H3N2) × A/Bel/42 (H1N1); Jap_H-Bel_N (H2N1), a genetic reassortant of A/Jap/305/57 (H2N2) × A/Bel/42; $Shear_H$-Bel_N (H6N1), a genetic reassortant of A/Shearwater/E. Aust/72 (H6N5) × A/Bel/42; and PR8 (A/Puerto Rico/8/34) (H1N1) (Mt. Sinai strain). BEV, KEN, MEG, V67, V127, and V152 are MAb-selected escape mutants of $Mem71_H$-Bel_N virus which bear single amino acid substitutions in the

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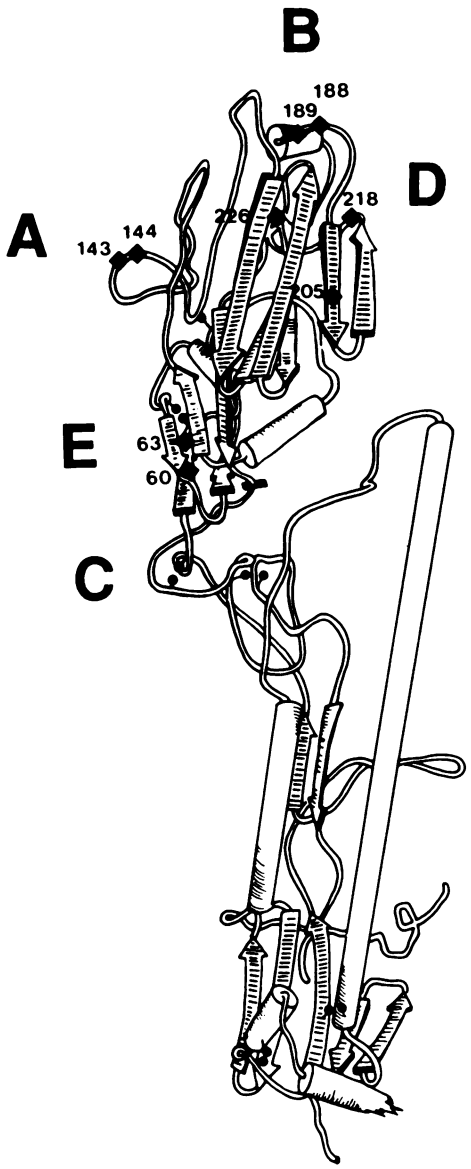

FIG. 1. Schematic diagram of the influenza virus HA monomer (H3 subtype) showing the location of the major antigenic sites (A to E) (36, 37). The monomer was drawn by Hidde Ploegh and is reproduced with permission of Macmillan Magazines Ltd., London (41).

HA molecule; in BEV, KEN, and MEG the substitution is in antigenic site A of HA (38), and in V67, V127, and V152 the substitution is in antigenic site E of HA (4). Type B influenza virus B/Hong Kong/2/75 was also used. Viruses were grown in the allantoic cavity of 10-day-old embryonated hen eggs and purified from the allantoic fluid by precipitation with polyethylene glycol (1), followed by sedimentation through 20 to 60% sucrose gradients. Hemagglutination assays were performed with chick erythrocytes by the method of Fazekas de St. Groth and Webster (7).

MAbs. The characteristics and sources of the anti-HA MAbs used are listed in Table 1. The MAbs were all of BALB/c origin and reacted with Mem71 HA (H3 subtype). The antigenic site against which each MAb was directed was inferred from its pattern of reactivity with a panel of MAb-selected escape mutants of $Mem71_H$-Bel_N virus (Table 1, footnote *c*). The five anti-HA MAbs used for the production of anti-Id antibodies (MAbs 36, 40, 170, 244, and 261) were all neutralizing antibodies of the immunoglobulin G2a (IgG2a) subclass and were derived from separate fusions. IgG was purified from ascitic fluid by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Ey et al. (6). MAb 14-4-4 (IgG2a, anti-$I\text{-}E^{k,d}$ [28]) was used as a control MAb.

TABLE 1. Characteristics of anti-HA MAbs

MAb[a]	Isotype	Antigenic site of epitope on the HA molecule[b]	Amino acid residues known to be implicated[c]
244	IgG2a	A	143, 144
95	IgG2b	A	143, 144
106	IgG2b	A	143, 144
170	IgG2a	B	188, 189
514	IgG1	B	188, 189
508	IgG1	B	188, 189
261	IgG2a	B/D	188, 189, 205, 218
207	IgG1	B/D	188, 189, 205, 218
203	IgG1	B/D	188, 189, 218
A21	IgG1	B/D	189, 205, 218
40	IgG2a	D	205, 218, 226
221	IgG2b	D	205, 218, 226
36	IgG2a	E	60, 63
67	IgG1	E	60, 63
127	IgG2a	E	60, 63
152	IgG2b	E	60, 63

[a] MAbs against which anti-Id antibodies were raised in this study are shown in boldface type. Hybridomas secreting MAbs 244, 514, 508, 203, 261, 207, 40, and 36 were made by J. M. Murray and L. E. Brown of this laboratory; MAbs 95, 106, 170, 221, 67, 127, and 152 were made by P. A. Underwood, Commonwealth Scientific and Industrial Research Organisation Division of Biotechnology, North Ryde, New South Wales, Australia; and MAb A21 was made by W. Gerhard, Wistar Institute, Philadelphia, Pa.

[b] Letters refer to the antigenic sites on influenza A virus HA of the H3 subtype, as defined by Wiley et al. (39, 40) (Fig. 1).

[c] The binding site of each MAb was inferred from its pattern of reactivity with a panel of escape mutants of $Mem71_H$-Bel_N virus selected by growth in the presence of neutralizing anti-HA MAbs or horse serum. The numbers denote the amino acid position in HA_1 at which a substitution adversely affected binding of the MAb. The mutant viruses used were V67 (Asp-60 → Val), V152 (Asp-63 → Tyr), and V127 (Asp-63 → Asn) (4) (the substitution in V127 creating a potential glycosylation site); KEN (Pro-143 → Thr), BEV (Pro-143 → Ser), MEG (Gly-144 → Asp), IAN (Asn-188 → Asp), DOUG (Gln-189 → His), DON (Ser-205 → Tyr), and TED (Gly-218 → Trp) (20, 26, 38); and a horse serum-resistant mutant of $Mem71_H$-Bel_N (Leu-226 → Gln). Data are from reference 4; J. M. Murray, Ph.D. thesis, University of Melbourne, Melbourne, Victoria, Australia, 1988; and this study.

Production and purification of anti-Id antibodies. Outbred New Zealand rabbits were injected subcutaneously and intramuscularly with 500 μg of MAb IgG in Freund complete adjuvant (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia). Two boosts identical to the primary dose were given on days 21 and 35. Sera were collected 7 to 10 days after the last injection, and the presence of anti-Id antibodies was determined by a radioimmunoassay (RIA) as described below. Antisera were subsequently made specific for Id by passage over Sepharose columns to which had been coupled normal mouse immunoglobulin [prepared by precipitation of normal mouse serum [NMS] with 40% $(NH_4)_2SO_4$]. The removal of antibodies directed against constant-region determinants on mouse immunoglobulin was monitored by the failure of the absorbed sera to bind in an enzyme-linked immunosorbent assay to wells coated with the control MAb

14-4-4. The IgG fraction of each absorbed anti-Id serum was then isolated by affinity chromatography on protein A-Sepharose (12), and the IgG was concentrated by ultrafiltration with a type CF25 membrane cone (Amicon Corp., Lexington, Mass.).

RIAs. All assays were carried out in 96-well polyvinyl microdilution plates (Dynatech, Sydney, New South Wales, Australia) at room temperature. Wells were coated overnight with 20 μl of protein in phosphate-buffered saline (pH 7.4) and incubated for 1 h with 100 μl of phosphate-buffered saline containing 10 mg of either bovine serum albumin or gelatin per ml to saturate the protein-binding capacity of the wells. Between subsequent steps of the assay, wells were washed with phosphate-buffered saline containing 0.05% Tween 20. Antibodies (unlabeled or ^{125}I labeled) were added in 20-μl volumes, the diluent being phosphate-buffered saline containing 0.05% Tween 20 and 5 mg of either bovine serum albumin (BSA_5-PBST) or gelatin per ml. The amount of radioactivity bound to individual wells was determined in a gamma counter. IgG preparations were iodinated by a modification (16) of the chloramine-T method of Greenwood et al. (13). The assays used were as follows.

(i) Assay of antiviral antibody. Wells were coated with influenza virus at 1,000 hemagglutinating units per ml. Serial dilutions of mouse serum or ascitic fluids were incubated in the wells overnight, and bound antibody was detected by the addition of 100,000 cpm of ^{125}I-labeled rabbit anti-mouse IgG (Dako Immunoglobulins, Glostrup, Denmark) for 4 h.

(ii) Assay of anti-Id antibody in rabbit antisera. The assay was based on that developed by Nepom et al. (25) and measured the binding of anti-Id antibody to the homologous ^{125}I-labeled MAb (Id). Wells were coated with 20 μl of protein A from *Staphylococcus aureus* (100 μg/ml; Pharmacia). Serial dilutions of unabsorbed anti-Id serum were added to the wells for 4 h and, after washing, the wells were incubated for 1 h or overnight with 2% normal rabbit serum in BSA_5-PBST to saturate any unoccupied immunoglobulin-binding sites on protein A. After further washing, the wells were incubated for 1 h with 20 μl of BSA_5-PBST containing 0.5% NMS to block rabbit antibodies directed against constant-region determinants on mouse immunoglobulin; without washing, 5 to 10 ng (100,000 cpm) of ^{125}I-labeled anti-HA MAb or control MAb 14-4-4 was added in 20 μl of the same diluent. After 3 h of incubation, the plates were washed and the ^{125}I-labeled MAb bound to each well was determined.

(iii) Id competition RIA. To detect Id expression on other immunoglobulin molecules, we modified the above-described assay so that protein A-coated wells received a dilution of the anti-Id serum determined previously to be limiting for the binding of ^{125}I-labeled MAb. Dilutions of the test competitor in BSA_5-PBST containing 0.5% NMS were added to the wells 1 h before the addition of labeled MAb. Results were expressed as the percent binding of labeled MAb in the presence versus the absence of competitor.

(iv) Antigen binding inhibition assay. The ability of anti-Id IgG preparations to inhibit the binding of different anti-HA MAbs to influenza virus was examined. Aliquots of ^{125}I-labeled anti-HA MAb (12,000 cpm/20 μl) were preincubated with BSA_5-PBST diluent or with serial dilutions of the corresponding rabbit anti-Id IgG or rabbit anti-mouse immunoglobulin IgG (Dako) for 1 h at room temperature before addition to $Mem71_H$-Bel_N virus-coated wells. After 4 h, the wells were washed and the amount of ^{125}I-labeled MAb bound to virus was determined. In other experiments, anti-Id IgG was preincubated with unlabeled anti-HA MAb or BALB/c anti-$Mem71_H$-Bel_N virus serum (used at a dilution giving less than maximal binding to the viral antigen), and subsequent binding of antibody to virus was monitored by the addition of 100,000 cpm of ^{125}I-labeled rabbit anti-mouse IgG.

Immunization of mice with anti-Id antibodies. Groups of six mice were immunized intraperitoneally with 40 μg of anti-Id IgG in Freund complete adjuvant and boosted in an identical manner 3 weeks later. Subsequent boosts with the same dose in Freund incomplete adjuvant were given intraperitoneally at approximately 1- to 2-month intervals, for a total of six injections. Mice were bled 12 days after each boost, and the sera were assayed for antiviral antibody by RIA.

Absorption of sera with influenza virus. Serum samples (150 μl of a 1/30 dilution) were incubated with 10^5 hemagglutinating units of purified influenza virus for 2 h at 4°C. The virus was removed by centrifugation in an air-driven centrifuge for 5 min at 85,000 × *g* (Beckman Instruments, Inc., Fullerton, Calif.).

Neutralization assays. Neutralization assays were performed by a modification of the allantois-on-shell method (8) Sera were heat inactivated (56°C, 30 min) before use. The medium used was RPMI 1640 supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 30 μg of gentamicin per ml, and 10% (vol/vol) normal allantoic fluid. Dilutions of antibody (100 μl, in duplicate) were preincubated with 20 infectious doses of $Mem71_H$-Bel_N virus for 1 h in the wells of a sterile 96-well flat-bottomed microdilution tray (Nunc, Roskilde, Denmark). Allantoic membrane (3 by 3mm, still attached to the egg shell) was added to the wells, and the plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 2 days, the growth of virus in each well was assessed by testing the culture supernatant for hemagglutinating activity. The endpoint was taken as the reciprocal of the highest dilution of serum that neutralized virus infectivity in 50% of the wells.

Antiviral serum. Antiserum to $Mem71_H$-Bel_N virus was raised by immunizing BALB/c mice intraperitoneally with 400 hemagglutinating units of $Mem71_H$-Bel_N virus in allantoic fluid, boosting the mice several months later with the same dose, and bleeding the mice after a further 2 weeks.

RESULTS

Anti-Id response in rabbits. Individual rabbits were immunized with one of the five anti-HA MAbs, 36, 40, 170, 244, and 261, which are directed against distinct or overlapping sites on the HA molecule (Table 1 and Fig. 1), and sera were tested for anti-Id antibodies as described in Materials and Methods. Figure 2 shows the data for rabbit antiserum to MAb 244. Antibodies against constant-region determinants of mouse immunoglobulin, detected by binding of an irrelevant ^{125}I-labeled IgG2a MAb, 14-4-4 (panel A), were completely blocked by the inclusion of 0.5% NMS in the latter steps of the assay (panel B), allowing the anti-Id response to MAb 244 to be clearly distinguished. Similar titers of anti-Id antibody were obtained for anti-Id sera raised against each of the other four MAbs.

Thereafter, antibodies reacting with normal mouse immunoglobulin were removed from all sera by absorption on a normal mouse immunoglobulin column, and the IgG fraction of each absorbed anti-Id preparation was isolated.

Specificity of anti-Id antibodies. The private or cross-reactive nature of the Id detected by each anti-Id serum was examined in an Id competition assay in which different anti-HA MAbs were tested for their ability to compete with

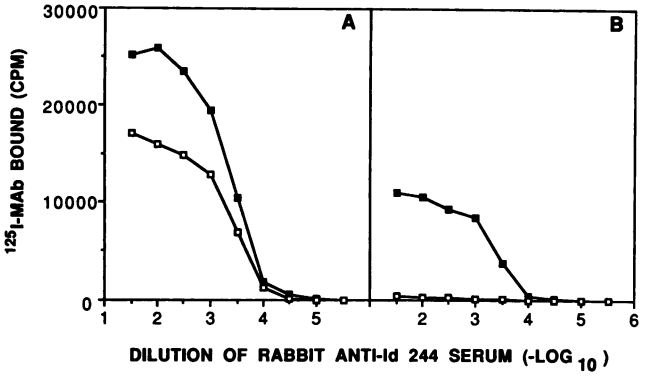

FIG. 2. Detection of anti-Id antibodies in serum from a rabbit immunized with MAb 244. Curves show the binding of ^{125}I-labeled MAb 244 (■) and ^{125}I-labeled control MAb 14-4-4 (□) to rabbit anti-MAb 244 antibodies in the absence (A) or presence (B) of NMS.

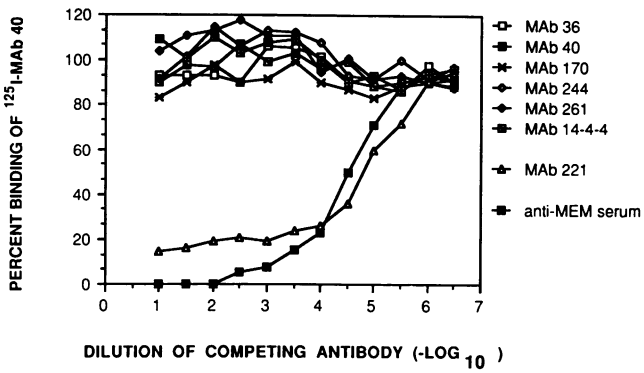

FIG. 3. Id competition RIA to detect the presence of the MAb 40 Id on other anti-HA MAbs. Curves show the binding of ^{125}I-labeled MAb 40 to anti-Id 40-coated wells in the presence of dilutions of various unlabeled competing anti-HA MAbs, control MAb 14-4-4, or BALB/c anti-Mem71$_H$-Bel$_N$ serum (anti-MEM serum).

a ^{125}I-labeled MAb for binding to its homologous anti-Id antibody. Each of the anti-Id sera detected a private specificity on the immunizing MAb, there being no cross-inhibition by any of the four heterologous anti-HA MAbs (Table 2).

To determine whether antibodies directed to the same antigenic site on HA share idiotypic determinants, we tested an additional 11 anti-HA MAbs of known specificity (Table 1) as competitors. Only one of these inhibited any of the Id/anti-Id interactions, despite the fact that all 11 recognized an epitope sharing at least two amino acid residues within the same general antigenic site as one of the five MAbs against which the anti-Id antibodies had been raised. However one MAb, 221, inhibited the binding of ^{125}I-labeled MAb 40 to anti-Id 40 by 80% (Fig. 3). MAbs 221 and 40 arose from different fusions and were of different isotypes but showed the same pattern of reactivity for mutant viruses, the epitopes of each being affected by amino acid substitutions in residue 205, 218, or 226 of HA.

Figure 3 also shows that an antibody bearing the Id of MAb 40 was not present at detectable levels in polyclonal BALB/c anti-Mem71$_H$-Bel$_N$ virus serum. The same was true for each of the other four Ids (data not shown). Thus, in this assay, each of the anti-Id preparations was directed predominantly to a private Id of the immunizing MAb which was not shared by MAbs binding to different antigenic sites but could be shared by MAbs of the same or very similar epitope specificity.

Inhibition by anti-Id antibodies of the binding of anti-HA antibodies to viral antigen. Each of the anti-Id IgG preparations completely inhibited the binding of the homologous ^{125}I-labeled MAb to Mem71$_H$-Bel$_N$ virus-coated wells, whereas a similar concentration of rabbit anti-mouse IgG gave minimal inhibition. Figure 4 shows the data for anti-Id 40. Since the inhibition was not the result of binding of the anti-Id preparations to virus (data not shown), these results indicate that each anti-Id serum recognizes at least some paratope-associated idiotopes on the immunizing MAb. The concentrations of anti-Id IgG required to inhibit homologous Id binding to virus by 50% were 0.4 μg/ml for anti-Id 170, 0.5 μg/ml for anti-Id 36 and anti-Id 40, 1 μg/ml for anti-Id 261, and 2 μg/ml for anti-Id 244.

In experiments in which ^{125}I-labeled anti-mouse immunoglobulin was used to detect the binding of unlabeled anti-HA MAbs to virus, inhibition by each anti-Id IgG was shown to be specific for the immunizing MAb. Cross-inhibition was observed on only two occasions, each involving a MAb directed against the same antigenic site as the immunizing MAb: (i) anti-Id 40 inhibited the binding of MAb 221 to virus (Fig. 5A), consistent with the strong idiotypic relatedness of MAbs 40 and 221 revealed by the Id competition assay (Fig. 3), and (ii) anti-Id 36, raised against the site E-specific MAb 36, weakly inhibited the binding of one of three additional site E-specific MAbs tested, MAb 67, but only when used at a high concentration (Fig. 5B).

None of the anti-Id antibodies, even at high concentrations, significantly inhibited the binding to virus of BALB/c anti-Mem71$_H$-Bel$_N$ serum, confirming the essentially private nature of the Ids recognized by these anti-Id sera.

Induction of antiviral antibodies in BALB/c mice by immunization with anti-Id antibodies. Groups of six BALB/c mice were immunized with rabbit anti-Id IgG or normal rabbit IgG. In all, six injections were given over a period of 8 months, and individual sera, taken 12 days after each boost, were assayed for antibody to Mem71$_H$-Bel$_N$ virus by RIA.

Mice immunized with normal rabbit IgG, anti-Id 40, anti-

TABLE 2. Specificity of anti-Id antibodies for the Id of the immunizing MAb as revealed by an Id competition RIA

Id/anti-Id interaction	Concn of the following competing MAb (μg of IgG/well) giving 50% inhibition of binding of ^{125}I-labeled MAb to anti-Id antibody-coated wells:					
	36	40	170	244	261	14-4-4 (Control)
^{125}I-MAb 36/anti-Id 36	0.002	>10	>10	>10	>10	>10
^{125}I-MAb 40/anti-Id 40	>10	0.003	>10	>10	>10	>10
^{125}I-MAb 170/anti-Id 170	>10	>10	0.001	>10	>10	>10
^{125}I-MAb 244/anti-Id 244	>10	>10	>10	0.001	>10	>10
^{125}I-MAb 261/anti-Id 261	>10	>10	>10	>10	0.001	>10

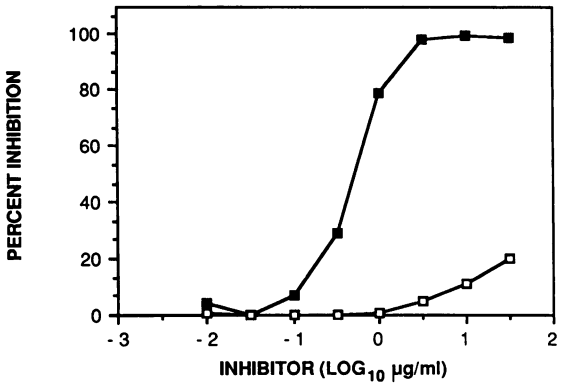

FIG. 4. Inhibition of binding of ^{125}I-labeled MAb 40 to Mem71$_H$-Bel$_N$ virus-coated wells by rabbit anti-Id 40 IgG (■) and by rabbit anti-mouse immunoglobulin IgG (□).

Id 170, or anti-Id 261 produced no antiviral antibody. However, five of six mice receiving anti-Id 36 and all six mice receiving anti-Id 244 developed significant levels of antiviral antibody after the third injection, and the titers rose in some but not all mice following further boosts. Figure 6 shows the data for sera taken after the sixth injection and includes, for comparison, the binding curve for BALB/c anti-Mem71$_H$-Bel$_N$ virus serum.

When tested for their ability to neutralize the infectivity of Mem71$_H$-Bel$_N$ virus by the allantois-on-shell method, two of the five positive sera from mice immunized with anti-Id 36 and four of the six sera from mice immunized with anti-Id 244 displayed activity. The neutralization titers were generally rather low (50 to 500), but in one mouse immunized with anti-Id 244 the titer (10,000) was as high as that of the standard BALB/c anti-Mem71$_H$-Bel$_N$ virus serum.

Antiviral response to anti-Id antibodies in other mouse strains. To determine whether the ability of anti-Id 36 and anti-Id 244 to induce antiviral antibodies was restricted to BALB/c mice (*H-2^d Igh^a*), we immunized inbred mice of three other strains differing from BALB/c in the major histocompatibility complex or in immunoglobulin heavy-chain allotype: CBA (*H-2^k Igh^a*), C57BL/10 (*H-2^b Igh^b*), and B10.D2 (*H-2^d Igh^b*). After three injections of either anti-Id 36 or anti-Id 244, the antiviral response was examined. With the exception of the rather weak response of CBA mice to anti-Id 244, the response of all three strains of mice was very similar to that of BALB/c mice (Fig. 7).

Fine specificity of the antiviral antibodies induced in BALB/c mice by anti-Id antibodies. The antiviral antibody induced by anti-Id 36 and anti-Id 244 in BALB/c mice was analyzed. The antibody was directed towards the HA molecule, since identical titration curves were obtained whether purified Mem71 HA or Mem71$_H$-Bel$_N$ virus was used as the capture antigen in the RIA (data not shown). To compare the fine specificity of this antibody with that of the original MAb used for immunization, we tested the antiviral serum from one mouse of each group for the ability to bind to MAb-selected escape mutants of Mem71$_H$-Bel$_N$ virus bearing

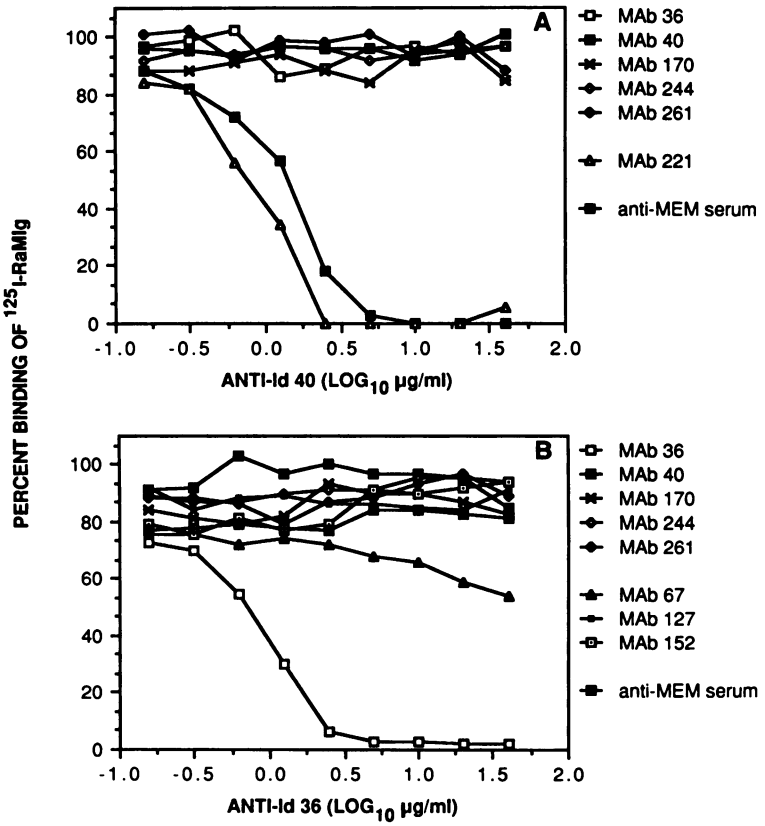

FIG. 5. Inhibition of binding of anti-HA MAbs and BALB/c anti-Mem71$_H$-Bel$_N$ serum (anti-MEM serum) to Mem71$_H$-Bel$_N$ virus-coated wells by dilutions of anti-Id 40 IgG (A) and anti-Id 36 IgG (B). Antibody binding was detected with ^{125}I-labeled rabbit anti-mouse immunoglobulin (^{125}I-RaMIg).

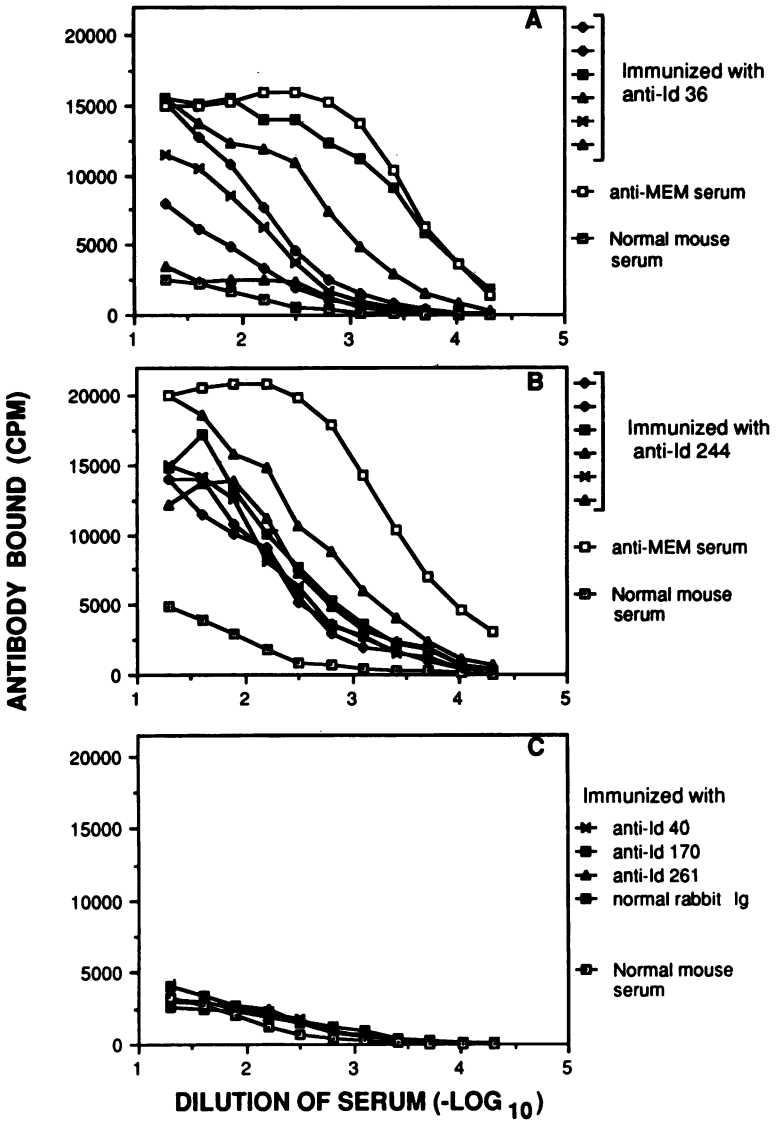

FIG. 6. Induction of antibody to $Mem71_H$-Bel_N virus in BALB/c mice by immunization with anti-Id antibodies. (A and B) Binding to $Mem71_H$-Bel_N virus-coated wells of the sera six individual mice immunized with anti-Id 36 IgG (A) or anti-Id 244 IgG (B) and, for comparison, the binding curves for BALB/c anti-$Mem71_H$-Bel_N virus serum (anti-MEM serum). For the anti-Id preparations that did not induce antiviral antibody (C) the curves shown represent the mean binding of the six sera from each immunization group. The sera were obtained from mice 12 days after the sixth immunization. Ig, Immunoglobulin.

single amino acid substitutions in either site A or site E of HA.

Antiviral antibody induced by anti-Id antibodies was directed to the same antigenic site on HA as the original immunizing MAb (Fig. 8). Antibody induced by anti-Id 36, like MAb 36 itself, failed to bind to mutant viruses altered in site E of HA (at residue 60 or 63) but was not affected by changes in site A (at residue 143 or 144) (Fig. 8A and C). Antiviral antibody induced by anti-Id 244 (Fig. 8B), like MAb 244 (Fig. 8D), showed the reciprocal properties, binding well to site E mutants but not to two of the three mutants altered in site A (Fig. 8B). Interestingly, in neither case did the antiviral antibody display a specificity identical to that of the immunizing MAb. Antibody induced by anti-Id 36 was more sensitive than MAb 36 itself to the substitution of Asp-63 with Tyr in mutant V152. Moreover, whereas MAb 244 was particularly sensitive to the change from Gly to Asp at residue 144 in mutant MEG, antibody induced by anti-Id 244 bound well to MEG but bound poorly to mutants KEN and BEV, which have changes in residue 143.

The specificity of the antibody induced by anti-Id 244 was analyzed further by absorbing the serum with $Mem71_H$-Bel_N virus. The antibody remaining in the supernatant after high-speed centrifugation bound only poorly to $Mem71_H$-Bel_N virus but still bound well to MEG (data not shown). Thus, this anti-(anti-Id 244) serum contained at least two populations of site A-specific anti-HA antibodies, one recognizing the parent Mem71 HA molecule and the other specific for mutant MEG.

Induction of antibodies against influenza viruses of other subtypes. None of the anti-HA MAbs used in this study cross-react with influenza A viruses of other subtypes or with influenza B viruses. Nevertheless, we were interested in determining whether, by immunizing mice with anti-Id

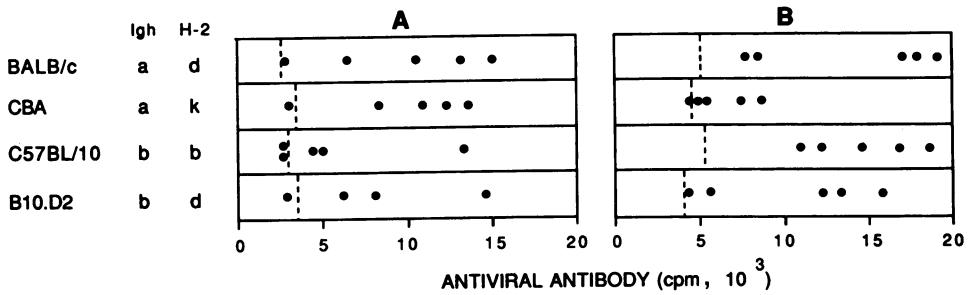

FIG. 7. Induction of antibody to $Mem71_H$-Bel_N virus in different mouse strains by immunization with anti-Id 36 IgG (A) or anti-Id 244 IgG (B). Mice received three injections of the appropriate anti-Id IgG in adjuvant on days 0, 22, and 50, as described in Materials and Methods. Sera were collected 17 days after the last injection and assayed for binding to $Mem71_H$-Bel_N virus by RIA. Each point represents the binding of an individual serum, tested at a 1/40 dilution. The dotted line represents the binding obtained with the same dilution of NMS from each strain.

antibodies, antibodies with broader reactivities for other influenza viruses could be raised. Antisera from mice immunized with anti-Id 36 or anti-Id 244 were therefore tested by RIA for their reactivity with influenza A viruses of heterologous HA subtypes [PR8 (H1N1), Jap_H-Bel_N (H2N1), and $Shear_H$-Bel_N (H6N1)] and with influenza B/Hong Kong virus.

The antibodies induced by anti-Id 244 did not bind to any of these viruses. However, sera from three of the six mice immunized with anti-Id 36 contained antibodies reactive with influenza virus of a heterologous HA subtype: two reacting with PR8 (H1N1) virus and one reacting with Jap_H-Bel_N (H2N1) virus (Table 3). The induction of these heterologous antibodies was similar to that of the antibodies reacting with $Mem71_H$-Bel_N virus, developing after the third injection of anti-Id 36, and in two cases the titers obtained were higher. In each instance, the heterologous activity was shown to represent a separate population of antibodies from those reacting with $Mem71_H$-Bel_N virus, since the removal of H3-specific antibody by absorption with $Mem71_H$-Bel_N

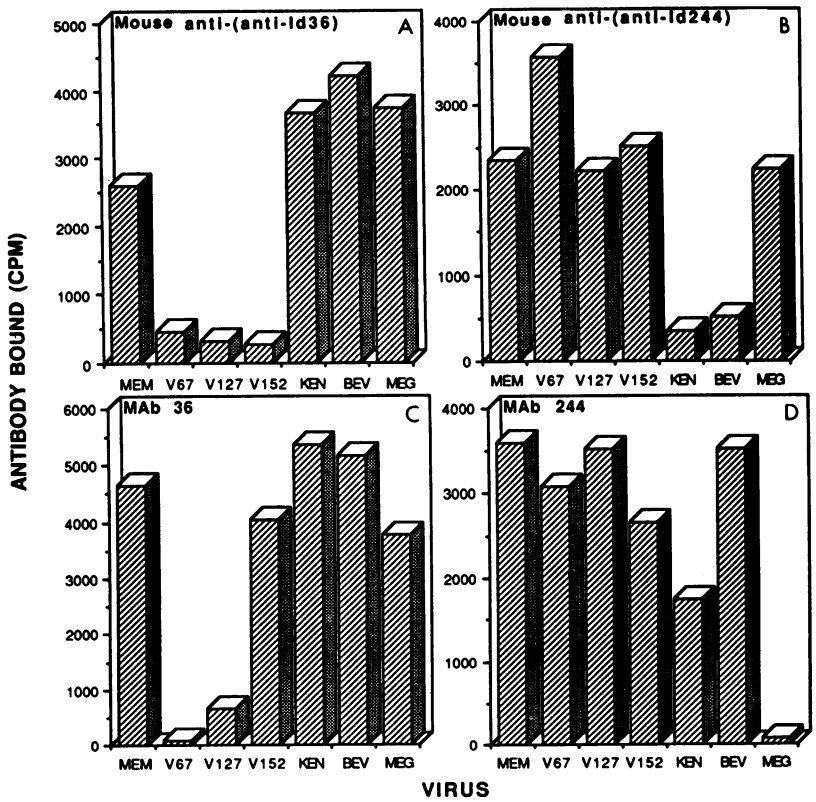

FIG. 8. Reactivity of BALB/c antiviral antibody induced by anti-Id 36 and anti-Id 244 with escape mutants of $Mem71_H$-Bel_N virus bearing single amino acid substitutions in site E or site A of HA. The histograms show the specific binding of a 1/320 dilution of anti-(anti-Id 36) serum (A) and anti-(anti-Id 244) serum (B) to each of the different virus-coated wells, corrected for the background binding obtained with the same dilution of BALB/c NMS. The binding of MAb 36 (C) and MAb 244 (D) is included for comparison. The viruses used were $Mem71_H$-Bel_N (MEM); site E mutants V67 (Asp-60 → Val), V127 (Asp-63 → Asn), and V152 (Asp-63 → Tyr); and site A mutants KEN (Pro-143 → Thr), BEV (Pro-143 → Ser), and MEG (Gly-144 → Asp).

TABLE 3. Induction by anti-Id 36 of antibodies to influenza A viruses of different subtypes[a]

Serum	Titer of antiviral antibodies against:				
	$Mem71_H$-Bel_N (H3N1)	Jap_H-Bel_N (H2N1)	PR8 (H1N1)	$Shear_H$-Bel_N (H6N1)	B/Hong Kong
1	350	<10	1,100	<10	<10
2	200	<10	<10	<10	<10
3	150	500	<10	<10	<10
4	15	<10	<10	<10	<10
5	100	<10	<10	<10	<10
6	200	<10	50	<10	<10
NMS	<10	<10	<10	<10	<10

[a] Sera were obtained from BALB/c mice 12 days after the third immunization with anti-Id 36 IgG. Sera 1 through 6 were from mice 1 through 6. Titers are expressed as the reciprocal of the serum dilution giving 50% maximum binding.

virus had no effect on the binding of the serum to the H1 virus (Fig. 9) or the H2 virus, respectively.

DISCUSSION

Previous idiotypic analyses of anti-influenza HA antibodies by others have identified the presence of both private and cross-reactive Ids (21, 24, 35). In the present study each of the five rabbit anti-Id sera was directed predominantly against a private Id of the immunizing anti-HA MAb, in that none of the five Ids could be detected in a pool of BALB/c antisera raised against $Mem71_H$-Bel_N virus or on the majority of other anti-HA MAbs tested. However, in two instances, partial idiotypic sharing was observed between the immunizing MAb and another anti-HA MAb directed against the same antigenic region of HA and derived from a different mouse, indicating that the Id may recur on antibodies having the same or similar epitope specificity.

When used as immunogens in mice, two of the five anti-Id preparations induced antiviral antibodies, namely, anti-Id 244 (raised against site A-specific MAb 244) and anti-Id 36 (raised against site E-specific MAb 36). The fine specificity of these antiviral antibodies was studied by examining their pattern of reactivity with mutants of $Mem71_H$-Bel_N virus and with influenza A virus strains of other HA subtypes. The antiviral antibodies induced were clearly directed to the same antigenic site on Mem71 HA as the immunizing MAb was. Thus, the antibodies induced by anti-Id 36 failed to react with mutants V67, V127, and V152, each of which has

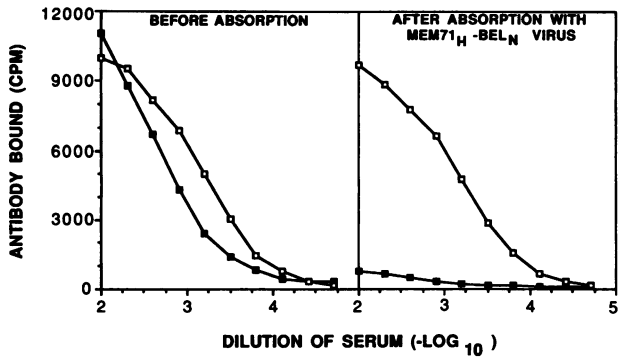

FIG. 9. Binding of serum from mouse 1 immunized with anti-Id 36 to wells coated with PR8 (□) or $Mem71_H$-Bel_N (■) virus before and after absorption of the serum with $Mem71_H$-Bel_N virus.

a single amino acid substitution in site E, whereas the antibodies induced by anti-Id 244 bound poorly to the site A mutants KEN (Pro-143 → Thr) and BEV (Pro-143 → Ser). Further analysis of selected antisera identified in each the presence of at least two distinct subpopulations of antiviral antibodies of differing specificity. A mouse immunized with anti-Id 244 produced not only site A-specific antibodies reactive with $Mem71_H$-Bel_N virus but also a second population, of a similar titer, of site A-specific antibodies that reacted with mutant virus MEG (Gly-144 → Asp) but not with $Mem71_H$-Bel_N virus itself. Thus, immunization with anti-Id antibodies led to a broadening of the antibody response, beyond that of the original MAb, to include antibodies that bound to a related virus but not to the parent virus against which the MAb was raised.

In mice immunized with anti-Id 36 this broadening of the antibody response extended to viruses of other HA subtypes. Thus, in addition to producing antibodies against site E of Mem71 HA some mice produced a separate population of antibodies binding to viruses of the H1 or H2 subtype. These heterologous antibodies appeared to be HA specific, since they failed to bind to other egg-grown viruses bearing A/Bel/42 (N1) neuraminidase and thus were not directed against neuraminidase or host cell-derived carbohydrate. If these antibodies arose by the same mechanism as the MEG-specific antibodies induced by anti-Id 244, they would be expected to bind to the equivalent of site E on the HA of the heterologous virus. We have been unable to examine the fine specificity of these antibodies, however, since we lack mutants of H1 and H2 subtypes with single amino acid substitutions known to be located in this region.

What is the mechanism of induction of antiviral antibodies by anti-Id 244 and anti-Id 36? One possibility, according to the "internal-image" hypothesis, is that these two anti-Id sera contain at least some antibodies of the Ab2β type. These Ab2β (which might represent only a small proportion of the total anti-Id antibodies present) would bear idiotypic structures whose conformation resembles the epitope on HA recognized by the original Ab1, i.e., within site A for MAb 244 and site E for MAb 36. At first site, the failure of anti-Id 244 and anti-Id 36 to react with BALB/c antisera to $Mem71_H$-Bel_N virus might argue against the presence of Ab2β, since these would be expected to behave like antigens and bind antiviral antibodies (27). This expectation may be valid for haptenic antigens or antigens with a single dominant antigenic site; however, the antibody response to HA is extremely diverse, there being five major antigenic sites (39) each comprising many overlapping epitopes. Indeed, Staudt and Gerhard (35) have estimated that BALB/c mice can make at least 1,500 paratypically distinct antibody molecules to the HA of PR8 virus. As discussed by Roitt et al. (30), the concept of internal image does not imply that the Ab2 molecule carries a structure resembling the entire antigenic site. Rather, it represents an image only of the particular epitope and perhaps only of the contact residues within the epitope seen by Ab1. Thus, an individual Ab2β molecule would react only with those antibodies that use a particular set of contact residues to bind the antigen. Such antibodies would represent only a proportion of those made against a particular antigenic site and would be rare in polyclonal antiviral serum.

The induction by anti-Id 244 and anti-Id 36 of antibodies against certain heterologous influenza viruses could be explained in terms of fidelity of the internal image. Since most Ab2β are unlikely to carry a perfect replica of the antigenic epitope, their use as immunogens would be expected to

induce not only antibodies with paratopes similar to Ab1 but also antibodies with related paratopes that fit the internal image but do not necessarily fit the antigenic epitope. Among the latter antibodies may be some that bind to an analog of the native epitope that is found in other influenza virus strains, e.g., in the case of antibody induced by anti-Id 244, to site A of MEG rather than of Mem71 HA itself.

Alternatively, the effective immunogen in the anti-Id sera giving rise to antiviral antibodies might not be Ab2β but rather an antibody of the Ab2α type directed against a framework Id of Ab1 that tends to be found in association with paratopes of a particular specificity (30). Immunization with these Ab2α would be expected to activate Id-positive B-cell clones, at least some of which produce antigen-specific antibodies. The production of antibodies with related but distinct fine specificities for the antigen could be explained by somatic mutation which accompanies clonal expansion during the immune response (22, 35). Both anti-Id 244 and anti-Id 36 induced antiviral antibodies in mice of the Igh^b as well as the Igh^a haplotype. Therefore, if the mechanism of induction involves Ab2α, the Ids concerned must be expressed by immunoglobulin molecules of both a and b heavy-chain allotypes.

The HAs of different subtypes of influenza A viruses are antigenically distinct. The induction by anti-Id 36 of antibodies specific for influenza viruses of the H1 or H2 subtype was therefore of particular interest. As mentioned earlier, we do not know whether these heterologous antibodies, like the Mem71 (H3)-specific antibodies, are specific for site E of the corresponding HA molecule; if so, they could arise by one of the two mechanisms described above. Alternatively, they may be directed to other sites on HA, and their mechanism of induction may be different from that of the Mem71-specific antibodies. One possibility would involve stimulation by Ab2α of B-cell clones bearing a cross-reactive (regulatory?) idiotope of the kind identified by Moran et al. (24) on anti-HA antibodies of differing specificity, including antibodies specific for H1 and H3 subtypes, and present also on a high proportion of human anti-influenza virus antibodies (34). However, immunization of mice with a syngeneic MAb against one such cross-reactive interspecies idiotope did not induce antiviral antibodies, although a marginal effect in priming for a subsequent anti-PR8 (H1N1) response was observed (32).

We hope to be able to gain further insight into the mechanism of induction of antibodies against both homologous and heterologous virus subtypes by using anti-Id MAbs that we have recently raised against MAbs 36 and 244. Work is also in progress to determine whether immunization with anti-Id antibodies (singly or in combination) has any protective effect against a subsequent influenza virus infection.

ACKNOWLEDGMENTS

We thank Mary Jones for excellent technical assistance and Julie Murray, Lorena Brown, and David Jackson for stimulating discussions.

This work was supported by Project Grant 840626 from the National Health and Medical Research Council of Australia.

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