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Anti-Idiotypic Antibodies Induce Neutralizing Antibodies to Rabies Virus Glycoprotein

KEVIN J. REAGAN, WILLIAM H. WUNNER,* TADEUSZ J. WIKTOR, AND HILARY KOPROWSKI
The Rabies Research Unit, The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

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Rabbit anti-idiotypic antibodies (αId Ab) were prepared against five murine monoclonal antibodies (mAb) specific for the rabies virus glycoprotein. Four of the mAb were directed against three known, type-specific, neutralizing sites on the glycoprotein, and the other mAb was directed against a topographically uncharacterized, nonneutralizing epitope. An absence of significant cross-reactivity among the αId Ab for heterologous mAb suggested that the αId Ab were highly specific for unique variable region determinants. The binding of three of the five αId Ab to their homologous mAb could be inhibited by rabies virus-soluble glycoprotein, suggesting that the αId Ab possessed subpopulations similar or adjacent to the antigen-binding site of the mAb. Two of the five αId Ab injected into mice elicited a specific virus-neutralizing antibody response. Mechanisms to account for the induction of the virus-neutralizing antibody by αId Ab are discussed.

The sequence of amino acids in the variable regions of both heavy (V_H) and light (V_L) chains of immunoglobulin produces a conformation in the antigen-binding site that allows interaction of the antibody with a specific antigen. Injection of immunoglobulin into a heterologous host animal gives rise to anti-xenotypic antibodies (specific for species), anti-isotypic antibodies (specific for the class of antibody), and anti-idiotypic antibodies (αId Ab; specific for the variable region). Two functional classes of αId Ab can exist, one which reacts with the antigen-combining site and another which is localized on the V_H chain or the V_L chain or both (framework determinants) (15).

Heterologous αId Ab have been useful in studies of immunodominance (2, 22), antibody diversity (30), and cell surface binding (24, 27). The operational theory in the latter type of studies is that a subset of the αId Ab represents a conformational homolog of the antigen to which the idiotype binds and thus exhibits binding specificity similar to this antigen.

We investigated the abilities of αId Ab made against various monoclonal antibodies (mAb) to reconstruct the epitopes of rabies virus glycoprotein. This glycoprotein is responsible for many of the important biological properties of rabies virus, including the induction of virus-neutralizing antibodies (VNA) (8), the site of antigenic drift (19, 35), the induction of cell-mediated immunity (33), and attachment to cell surface receptors (25; Wunner and Reagan, manuscript in preparation). Precisely which portion of the glycoprotein is responsible for each of these functions is unknown at present. Lafon et al. (19) have recently presented a functional epitope map for the challenge virus standard (CVS) strain of rabies virus glycoprotein, which suggests the existence of at least three major antigenic sites for type-specific VNA.

In this study we prepared αId Ab against five anti-glycoprotein mAb (αG mAb). These αG mAb were directed against the known functionally defined antigenic sites of rabies virus glycoprotein, and all but one strongly neutralized virus infectivity. The derived αId Ab were specific for the idiotypic regions of their respective hybridoma-derived antibodies, and two of the five elicited VNA in mice. The VNA produced were specific for rabies virus and failed to neutralize a variant virus selected by the original αG mAb. This demonstrates that an αId Ab can mimic the antigenic structure of a viral protein well enough to induce a neutralizing antibody.

MATERIALS AND METHODS

mAb. Anti-rabies virus glycoprotein mAb 509-6, 101-1, 507-1, and 719-3 have been described previously (19, 35) (Table 1). αG mAb 1104-2 was obtained from an additional fusion of splenocytes from rabies virus-immunized BALB/c mice with variant 653 of P3x63Ag8 mouse myeloma cells (17) by methods described elsewhere (34). The anti-glycoprotein-secreting hybridoma cells were selected and cloned by limiting dilution, and ascites fluids were prepared as previously described (34). Anti-rabies virus nucleocapsid mAb 515-3 (γ_{2a} isotype) and 389-1 (γ_1 isotype)

TABLE 1. Characterization of anti-rabies virus glycoprotein mAb used for production of αId Ab

αG mAb	Antigenic site[a]	Isotype[b]	Titer of mAb ascites fluid as determined by:	
			RIA[c]	Neutralization[d]
509-6	I	γ_{2a}	≥600,000	24,300
101-1	IIb	γ_1, γ_{2a}[e]	≥600,000	60,000
719-3	IIc	γ_{2a}	24,300	24,300
507-1	IIIb	γ_1	≥600,000	≥600,000
1104-2	?	γ_1	81,900	<300[f]

[a] Determined from the epitope map of Lafon et al. (19).

[b] Determined by the RIA, using specific rabbit anti-mouse isotype serum (12).

[c] RIA with strain ERA virus as the target antigen. The endpoint was determined to be the reciprocal of the highest dilution yielding reactivity that was 10% above the background level.

[d] Virus neutralization using a 20-h rapid fluorescent focus inhibition test with strain ERA virus. The neutralization endpoint was determined as the reciprocal of the highest serum dilution which reduced the number of infected cells by 50%.

[e] For γ_1, myeloma protein from P3x63Ag8 cells.

[f] The corresponding tissue culture fluid was also negative for VNA at a 1:9 dilution.

were isolated by similar techniques from Kelev virus-immunized BALB/c mice.

Viruses. Stocks of rabies virus strains ERA and CVS were propagated in BHK-21 cells by standard methods (31). The rabies virus antigenic variants ERA RV194-2 and RV509-6 have been described previously (11) and represent viruses which are resistant to neutralization by αG mAb 194-2 and 509-6, respectively. Rabies soluble glycoprotein (G_s) was purified from virion-depleted culture fluids by immunoadsorbent chromatography, as previously described (10).

Antibody purification. All αG mAb were purified from ascites fluids. Antibodies of the γ_{2a} isotype were diluted approximately 1:30 with 0.2 M Britton-Robinson buffer (pH 8.0), which consisted of 0.029 N citric acid monohydride, 0.058 N potassium phosphate (monobasic), 0.029 N barbital, and 0.087 N boric acid (30). This preparation was passed through a 0.45-μm membrane filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and then over a protein A-Sepharose 4B adsorption column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The column was washed with 30 ml of Britton-Robinson buffer (pH 8.0) before antibody was eluted with Britton-Robinson buffer at pH 3.0. αG mAb of the γ_1 isotype were first precipitated with sodium sulfate at a final concentration of 18% (wt/vol). The immunoglobulin fraction was dissolved and dialyzed in Britton-Robinson buffer (pH 8.0) and then passed over the protein A-Sepharose column as described above. Immunoglobulin eluted from the column was detected by a radioimmunoassay (RIA), using strain ERA virus as the antigen (9). The antibodies were concentrated by vacuum dialysis against phosphate-buffered saline (pH 7.4). Protein concentrations were determined by using bovine serum albumin as the standard (6).

αId Ab. αId Ab were prepared by the method of Staudt and Gerhard (30). Briefly, female New Zealand white rabbits were injected subcutaneously in multiple sites along the mammary chain with 300 μg of protein A-Sepharose-purified mAb emulsified in Freund complete adjuvant. Two intramuscular boosters of 100 μg of antibody in phosphate-buffered saline were given on days 7 and 30, and sera were collected 10 days later. Each anti-idiotypic antiserum was made specific for idiotype regions by passage over a Sepharose 4B column to which γ_1 or γ_{2a} anti-rabies nucleocapsid mAb were coupled. The effluent from such columns contained the antibodies that were reactive with the idiotypic determinants. Antibodies to constant regions were eluted from the anti-nucleocapsid immunoadsorbent column with 0.1 M diethylamine (pH 11.5). Immunoglobulin G (IgG) from each αId Ab was isolated by protein A-Sepharose chromatography as described above.

RIA. A solid-phase RIA was used to measure the binding of αId Ab to fixed mAb. Individual mAb were diluted (1:1,000 to 1:16,000, depending on the ascites fluid concentration) in carbonate-bicarbonate buffer (pH 8.9), and 25-μl portions were added to wells of polyvinyl microtiter plates (Dynatech Laboratories). These antibodies were allowed to dry in the wells by overnight incubation at 37°C. The free binding sites on the wells were blocked for at least 1 h with 10% agamma horse serum (GIBCO Laboratories, Grand Island, N.Y.) dissolved in phosphate-buffered saline containing 0.08% sodium azide. Dilutions (25 μl) of αId Ab were added, and after 1 h at room temperature the plates were washed extensively. Bound αId Ab were detected by adding 25 μl (30,000 cpm) of ^{125}I-labeled goat anti-rabbit IgG (Cappel Laboratories, Downingtown, Pa.) labeled by the iodogen method (21) and incubating the preparations for an additional 1 h at room temperature. All dilutions of αId Ab or radiolabeled probe were made in 10% agamma horse serum dissolved in phosphate-buffered saline containing 0.08% sodium azide. The plates were washed free of unbound probe, and the radioactivity bound to individual wells was measured with a gamma counter. All data represent the mean values of duplicate or triplicate assays.

Virus neutralization assay. The levels of VNA in immunized mice were determined by a modification of the rapid fluorescence focus inhibition test (29). Serial twofold dilutions of mouse serum were prepared in Microtiter II plates (50 μl/well; Falcon Plastics, Oxnard, Calif.) and incubated for 1 h at 37°C with an equal volume of virus containing 10^4 PFU/50 μl. After incubation, 50 μl of freshly trypsinized BHK-21 cells (2×10^6 cells per ml) was added to each well, the preparations were mixed, and 10-μl samples of the serum-virus-cell mixture were transferred (in dupli-

cate) into wells of Terasaki plates (Falcon Plastics). After 20 h of incubation, the plates were rinsed with phosphate-buffered saline and then with 80% (vol/vol) acetone in distilled water and fixed for 30 min in 80% acetone at room temperature. The plates were dried, and the cells were stained for 30 min at 37°C with 5 μl of fluorescein-conjugated anti-rabies nucleocapsid antibody of rabbit origin per well (32). Approximately 40% of the cells in the control wells (containing virus but no antibody) contained rabies virus-specific inclusions. The endpoint of virus neutralization was defined as the reciprocal of the highest serum dilution that was capable of reducing the number of rabies virus-infected cells by 50%.

Anti-αId Ab. Groups of four, ICR mice were inoculated subcutaneously with 40 μg of protein A-Sepharose-purified anti-idiotypic IgG (αId IgG) emulsified in Freund complete adjuvant per mouse. Subcutaneous booster inoculations were administered on days 7 and 32; each of these contained 40 μg of αId IgG in Freund incomplete adjuvant. At 5 days after the last booster inoculation, the animals were bled via the retroorbital plexus, and the pooled sera were checked for VNA. As a control, another group of mice received immunizations with protein A-Sepharose-purified normal rabbit IgG.

RESULTS

Preparation of αId Ab. Five αG mAb were selected from a large panel of hybridomas (19) to produce αId Ab in rabbits. These mAb were chosen because of their isotypes (enabling purification by protein A-Sepharose chromatography) and binding sites on the rabies virus glycoprotein, as defined by functional epitope mapping (19). Four of the mAb strongly neutralized viral infectivity. One other mAb, αG mAb 1104-2, was selected because it demonstrated excellent binding to strain ERA virus but poor neutralization. The characteristics of the mAb selected are summarized in Table 1. Each mAb was purified from ascites fluid and injected into rabbits to produce a hyperimmune serum as described above. Each hyperimmune rabbit serum was extensively adsorbed against purified mAb having the identical isotype but unrelated specificity (anti-rabies nucleocapsid mAb 515-3 or 389-1) to eliminate antibodies to constant region determinants. The success of this procedure was monitored by the RIA, and typical results are shown for αId Ab 101-1 in Fig. 1. Before adsorption, the antiserum to αG mAb 101-1 reacted equally well with either αG mAb 101-1 or an anti-nucleocapsid mAb having an identical isotype, suggesting a strong reaction to common determinants. Immunoglobulin which was eluted from the anti-nucleocapsid immunoadsorbent column reacted strongly with both antigens, indicating that it contained the cross-reactive material. In contrast, the material that

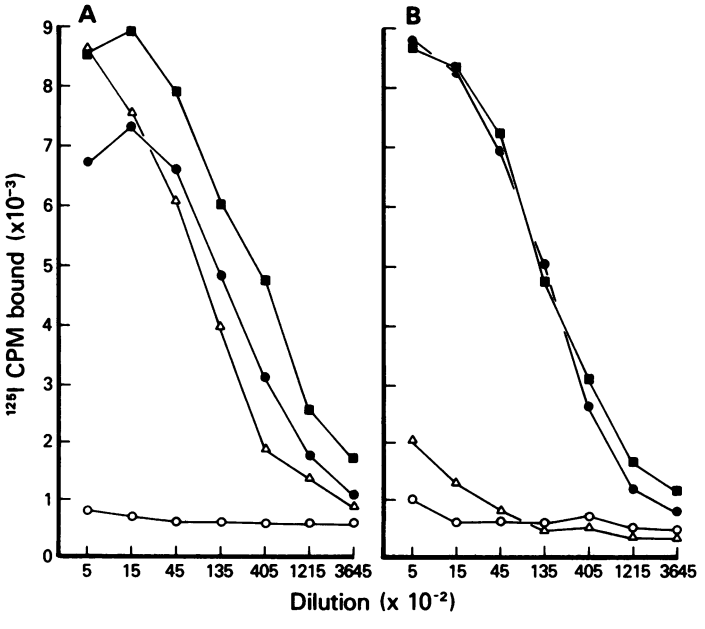

FIG. 1. Titration of αId Ab 101-1 by RIA, using αG mAb 101-1 (α-glycoprotein) (A) or α-nucleocapsid mAb 515-3 (having the identical isotype) (B) as the antigen. Dilutions of mAb (101-1, 1:6,000; 515-3, 1:1,500) were dried to soft plastic wells. Threefold serial dilutions of sera (prebleed serum [○], hyperimmune rabbit serum before adsorption with α-nucleocapsid mAb [■], immunoglobulin eluted from α-nucleocapsid immunoadsorbent column [●], αId Ab [△]) were added in duplicate to wells. The plates were washed extensively, and the amounts of bound antisera were determined by using a ^{125}I-labeled goat anti-rabbit antibody probe.

TABLE 2. Titration of αId Ab on homologous and heterologous mAb[a]

αG mAb (antigen)	αId IgG				
	509-6	101-1	719-3	507-1	1104-2
509-6	40,500	1,500	<500	<500	<500
101-1	<500	40,500	<500	<500	<500
719-3	1,500	<500	121,500	500	1,500
507-1	<500	<500	<500	13,500	<500
1104-2	<500	500	500	500	121,500

[a] The titer of each αId IgG was determined by solid-phase RIA, using mAb from ascites fluid as the antigen, as described in the text. Titers are expressed as reciprocals of the highest dilutions yielding counts at least 10% above background levels. Ascites fluids were diluted in carbonate-bicarbonate buffer as follows: 509-6, 1:3,000; 101-1, 1:6,000; 719-3, 1:6,000; 507-1, 1:6,000; 1104-2, 1:16,000.

passed through the column reacted only with the αG mAb and therefore contained the αId Ab. The reactivity of αId Ab 101-1 with nonidiotypic determinants was the maximum observed with any of the αId Ab preparations and was considered to be negligible.

Characterization of αId Ab. To demonstrate the specificity of the αId Ab and to determine whether cross-reactive idiotypes existed among the various αG mAb, each αId Ab preparation was titrated against homologous and heterologous mAb in an RIA. Table 2 clearly shows that each αId Ab was specific for its homologous mAb.

Although each αId Ab reacted only with the immunizing mAb, the proportion of the αId Ab response which was a response to the framework or antigen-binding sites of the mAb was not clear. Therefore, we devised a competition RIA to test the ability of rabies virus glycoprotein to prevent the interaction between idiotype and anti-idiotype (7, 28). The rabies G_s was used as the competing antigen. As shown in Fig. 2, the binding of three of the five αId Ab to their corresponding αG mAb was inhibited by G_s. Maximum inhibition varied from 20 to 50% with as much as 6 μg of G_s per ml, but at least a 15% reduction was observed with as little as 0.75 μg of G_s per ml. The inability to inhibit the binding of αId Ab totally was a clear indication that both framework and antigen-combining site specificities were present in three of the polyclonal αId Ab sera. The absence of any significant binding inhibition by G_s for αId Ab 101-1 or αId Ab 719-3 suggested a minor population or no population of antigen-binding specificities in these sera.

Preparation of anti-αId Ab. αId Ab reactive with the antigen-combining sites of αG mAb may contain subpopulations which mimic the viral epitopes recognized by these αG mAb. Therefore, inoculation of animals with αId Ab may induce antibodies which react with native rabies virus glycoprotein. Accordingly, protein A-Sepharose-purified αId IgG was used to immunize mice, and the derived sera were tested for neutralization of rabies virus by the rapid fluorescent focus inhibition test (Table 3).

Significant VNA titers were generated against rabies virus strain ERA in mice immunized with αId Ab 509-6 and with αId Ab 1104-2. The three other anti-αId Ab sera failed to neutralize strain ERA virus. Control experiments showed that mouse anti-normal rabbit IgG, as well as the αId Ab used for immunization, had no neutralizing activity. Furthermore, preincubation of anti-αId Ab 509-6 with αId Ab 509-6 but not normal rabbit serum removed the neutralizing activity. To test the specificity of the VNA generated, other rabies viruses were used in the neutralization assay. The pattern of reactivity of the αG mAb with these viruses should have been the

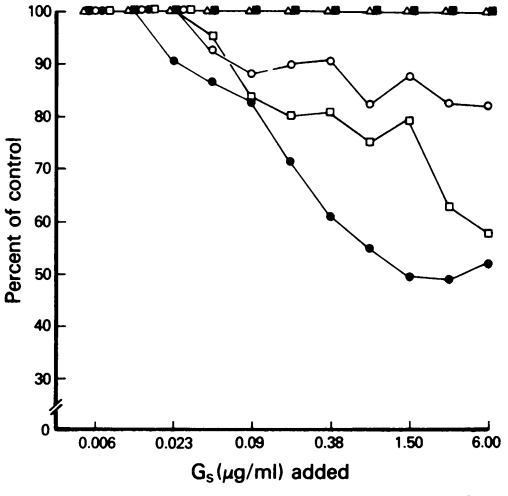

FIG. 2. Competition by G_s for interaction of αId Ab with homologous mAb. Pretitrated levels of mAb were incubated with serial twofold dilutions of G_s for 1 h before the addition of a standardized αId Ab dilution. The amount of αId Ab bound was probed as described in the legend to Fig. 1. Symbols: △, αId Ab 719-3; ■, αId Ab 101-1; ○, αId Ab 509-6; ●, αId Ab 507-1; □, αId Ab 1104-2.

TABLE 3. Neutralization of rabies virus strains by anti-αId Ab serum[a]

Mouse antiserum to:	Neutralization titer			
	Strain ERA			Strain CVS (parent)
	Parent	RV 509-6	RV 194-2	
Normal rabbit IgG	<4	<4	<4	<4
αId Ab 509-6	32	<4	64	64
αId Ab 101-1	<4	ND[b]	ND	ND
αId Ab 719-3	<4	ND	ND	ND
αId Ab 507-1	<4	ND	ND	ND
αId Ab 1104-2	128	<4	128	64
RIG[c]	16	16	32	32

[a] The reciprocal of the highest dilution of serum capable of reducing the number of infected cells by 50% was taken as the neutralization titer.

[b] ND, Not done.

[c] Human anti-rabies immunoglobulin was diluted to 0.2 IU/ml, and serial twofold dilutions were incubated with rabies virus as described in the text.

same as the pattern observed with the respective anti-αId Ab sera. Strain CVS of rabies virus bound both αG mAb 509-6 and αG mAb 1104-2 (data not shown), and Table 3 shows that strain CVS was neutralized by both anti-αId Ab 509-6 and anti-αId Ab 1104-2. On the other hand, a variant of strain ERA, RV 509-6, which possessed a mutation in the 509-6 epitope resulting in the loss of binding or neutralizing activity by αG mAb 509-6 (19), was not neutralized by anti-αId Ab 509-6. Another neutralization-resistant variant, RV 194-2, was effectively neutralized by anti-αId Ab 509-6. Previous results demonstrated that the antigenic sites recognized by αG mAb 509-6 and αG mAb 194-2 are totally independent (19). These data show that anti-αId Ab 509-6 reacts solely with the epitope which is recognized by αG mAb 509-6, and thus this epitope was simulated by αId Ab 509-6.

The level of complexity of the structural requirements for virus neutralization is demonstrated by the results obtained with anti-αId Ab 1104-2. αG mAb 1104-2 was originally chosen for this work because of its lack of neutralizing activity, yet anti-αId Ab 1104-2 contained VNA. Even though αG mAb 1104-2 bound well to RV 509-6 (data not shown), this variant virus was not neutralized by anti-αId Ab 1104-2. As expected, anti-αId Ab 1104-2 neutralized RV 194-2. A more direct test of anti-αId Ab 1104-2 neutralization specificity was not possible because nonneutralizing αG mAb 1104-2 could not be used to select resistant variant viruses. The titer of VNA in either group of mice was not sufficient to protect against a lethal intracerebral challenge with virus strain CVS.

DISCUSSION

Studies involving purified rabies virus glycoprotein or peptide fragments of glycoprotein have been hampered by hydrophobicity and destruction of native glycoprotein conformation. Thus, a soluble probe that imitates the secondary or tertiary conformation of glycoprotein in its native form would be of obvious benefit. αId Ab were prepared against various αG mAb with the intention of generating a population of internal image antibodies (16) which would mimic the epitope on native glycoprotein to which the αG mAb bound. Below we discuss the likelihood of such an event and also suggest a second mechanism for the induction of VNA.

The data in Fig. 1 and Table 2 indicate that the αId Ab obtained were highly specific for their homologous mAb. The absence of significant cross-reactions among the idiotypic regions of the mAb (Table 2) was not surprising since (i) the mAb selected bound to nonidentical areas on the glycoprotein epitope map (19) and (ii) other workers have found a low incidence of cross-reacting idiotypes among mAb directed to the same antigenic site but derived from different fusions (30). From Fig. 2, which demonstrates the competition between αId Ab and G_s for binding to mAb, we conclude that only a fraction of the αId Ab actually possessed G_s-modifiable reactivity with mAb (the operative definition of antigen-binding site specificity). We realize that this competition may be the result of steric inhibition rather than true conformational homology (20). Nevertheless, if or when an internal image antibody is generated, it should be structurally similar, if not identical, to the epitope bound by the original mAb and thus trigger the expression of idiotype-specific B-cell populations (3, 4). The fact that two αId Ab were able to induce VNA is consistent with the hypothesis that there was a conformation in the αId Ab which was similar to the viral epitope. It is clear, at least in the case of anti-αId Ab 509-6, that the VNA produced was identical to the monoclonal specificity of αG mAb 509-6. Like αG mAb 509-6, the VNA produced reacted only with the 509-6 epitope present on strain ERA, CVS, and RV 194-2 viruses; it did not react with RV 509-6.

Precisely why only two of five αId Ab were successful in eliciting a neutralizing response can only be a matter for speculation. It may be that of the three nonneutralizing antibody responses, αId Ab 101-1 and αId Ab 719-3 had only very minor proportions of their αId Ab repertoires directed against the antigen-binding site (Fig. 2). If this were the case, the chances of detecting a neutralizing response would be low. On the other hand, αId Ab 507-1 possessed the highest proportion of antigen-binding site specificities (Fig. 2), yet it failed to induce VNA. We

cannot rule out the possibility that immunization of mice with this αId Ab resulted in the stimulation of suppressor T cells (12), the deletion of B cells possessing the relevant idiotype (1, 23), or the induction of antibodies with too low an avidity to neutralize virus. Studies have shown that the induction or suppression of idiotope-positive B cells can depend upon the antibody class and the dose of αId Ab (12, 18). We also cannot rule out the possibility that αId Ab 101-1, αId Ab 719-3, and αId Ab 507-1 failed to induce VNA not because of the amount of internal image antibodies present, but rather because of an absence of B cells bearing the appropriate idiotopes in the repertoire of ICR mice.

Antigen mimicry by αId Ab does not explain all of our data, and indeed, the existence of internal image antibodies has been questioned (5, 20). If αId Ab 1104-2 had successfully reconstructed the epitope recognized by αG mAb 1104-2, one would expect it to induce a nonneutralizing antibody in mice similar to αG mAb 1104-2. Instead, anti-αId Ab 1104-2 possessed the highest VNA titer and a neutralization pattern similar to that of anti-αId Ab 509-6.

However, the hypothesis that the 509-6 and 1104-2 epitopes are related is unsupported by two lines of evidence. First, αId Ab 1104-2 did not cross-react with αG mAb 509-6 (Table 2), and therefore αId Ab 1104-2 was not identical to αId Ab 509-6. Second, αG mAb 1104-2 bound well to RV 509-6, which lacked the 509-6 epitope. Therefore, it is apparent, that αId Ab 1104-2 has some properties which distinguish it from the epitope recognized by mAb 1104-2.

It has been shown previously that mice treated in vivo with xenogeneic αId Ab can express an antibody found in their sera that can inhibit the interaction of idiotope with αId Ab (2, 3, 13). Studies with monoclonal anti-αId Ab have shown that the idiotope expressed by the anti-αId Ab can differ from the original idiotope, but these typically fail to bind antigen (4). Our finding of an alteration of binding specificity may suggest an alternative explanation for the induction of VNA. It is possible that the stimulation of idiotope-positive B cells may not depend on an encounter with an internal image antibody but rather on an encounter with an αId Ab specific for a framework sequence. This framework sequence may represent a cross-reactive idiotype or precursor B cells, which are preferentially utilized in the generation of antibodies specific for rabies virus glycoprotein. Upon clonal expansion and somatic diversification, the fine specificity of the secreted antibodies may vary (30). Since only a neutralizing antibody would be detected in the functional test used to assay the effectiveness of anti-αId Ab production, αId Ab 1104-2 derived from a non-neutralizing mAb would appear to have induced VNA. This argument is weakened, however, by the absence of detectable cross-reactive idiotypes among the mAb (Table 2).

Current studies are aimed at distinguishing between the internal image and framework hypotheses for induction of VNA by determining the usefulness of the αId Ab as cell surface probes, a particular function of internal image antibodies (24, 27). Sacks et al. (26) also have reported evidence for antigen-independent immunization in αId Ab derived from anti-*Trypanosoma* mAb. We believe that our findings support the idea that immunization with αId Ab can induce specific virus-neutralizing responses, as has been theorized by other workers (1, 14). Our findings also provide a basis for speculation on the incidence of naturally occurring αId Ab in humans treated with rabies immune globulin and its potential effect on the immune regulation network.

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