Characterization of an Ig V_H Idiotope That Results in Specific Homophilic Binding and Increased Avidity for Antigen[1]

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mAb against G_{D3} ganglioside demonstrate homophilic binding in which soluble anti-G_{D3} mAb bind, through the G_{D3} binding site, to a V_H idiotope (designated Id_{HOM}) on solid phase anti-G_{D3} mAb. In this way, homophilic binding provides a mechanism for amplifying the binding of mAb to cell surface G_{D3}. We show that serine 52a, within CDR2, is required for Id_{HOM} expression, homophilic binding, and high avidity binding to cell surface G_{D3}. Computer modeling based on the crystal structure of anti-G_{D3} mAb R24 showed serine 52a situated at the mouth of the G_{D3} binding pocket, but not directly involved with G_{D3} binding. Substitutions at position 52a predicted to maintain the G_{D3} binding pocket (e.g., threonine) resulted in the loss of Id_{HOM} expression and homophilic binding and markedly decreased binding to cell surface G_{D3}, but maintained low avidity G_{D3} binding as measured by ELISA. All other substitutions at position 52a were predicted to significantly distort the G_{D3} binding pocket and resulted in the loss of both homophilic binding and any detectable avidity for G_{D3}. We have structurally defined Id_{HOM} and conclude that this idiotope is not required for the G_{D3} binding pocket, but that the idiotope is necessary for homophilic binding, which is required for high avidity binding to cell surface G_{D3}. We speculate that selection of certain V_H genes may result in the expression of idiotopes that allow homophilic binding, and this may represent a general mechanism for increasing the avidity of Abs against T cell-independent Ags. *The Journal of Immunology,* **1996, 157: 1582–1588.**

Ag binding sites on Ig molecules are formed by three-dimensional conformations of amino acid residues within the heavy and light chain variable regions. Typically, the critical amino acids that contact Ag are situated within complementarity-determining regions (CDRs).[4] In addition to Ag binding sites, Ab variable regions can express binding sites for other molecules, such as B cell superantigens (1), Abs (2), and other members of the Ig supergene family (3, 4). These alternative binding sites, or epitopes, are usually distinct from the actual Ag binding site and may play a crucial role in the regulation of Ab production. Unique variable region epitopes, i.e., idiotopes, and anti-idiotypic Abs that bind to them have been hypothesized to play a role in activating or suppressing the B cell clone, leading to increases or decreases in the Ab concentration.

In addition to the regulatory role of idiotopes, we have considered the possibility that they could also provide a mechanism for increasing Ag binding avidity. We have observed that mAb directed against the G_{D3} ganglioside bind to themselves and to each other, a phenomenon termed homophilic binding (5). Initial studies demonstrated that homophilic binding does not occur in solution and that the target mAb expressing the homophilic binding epitope must be bound to a surface. It was also apparent that the homophilic binding epitope was expressed within the V_H region of anti-G_{D3} mAb, since the epitope was still expressed by variants in which the entire light chain had been replaced. Several lines of investigation pointed toward the model that the G_{D3} binding site of one mAb could bind the V_H epitope expressed on immobilized anti-G_{D3} mAb. We reasoned that homophilic binding may contribute significantly to the apparent avidity of mouse anti-G_{D3} mAb for G_{D3} by providing multivalent binding; mAb could bind to both G_{D3} and the homophilic binding epitope on the bound mAb, producing a cross-linked lattice of bound mAb.

Since all mouse anti-G_{D3} mAb tested to date express the V_H homophilic binding epitope, this epitope can be considered a shared or cross-reactive idiotope, which we designated Id_{HOM}. We hypothesized that Id_{HOM} is an example of an idiotope that increases the avidity of binding to Ag, and this may represent a mechanism common to mAb against other Ags as well. Here, we performed binding studies to determine whether homophilic binding is required for high avidity binding to G_{D3}. We also mapped Id_{HOM} within the V_H region of mouse anti-G_{D3} mAb and determined the structural relationship between Id_{HOM} and the G_{D3} binding site.

Materials and Methods

Cell lines

Mouse hybridomas R24 (IgG3), C5 (IgG3), MB3.6 (IgG3), K9 (IgM), and 11A (IgM) all secrete mAb against G_{D3} and have been described previously (5–7). Mouse-human chimeric R24 (chR24) consists of the R24 V_H and V_L domains ligated onto human IgG1(κ) constant regions (8, 9). HJM1, a gift from Dr. Lloyd Old, is a human lymphoblastoid cell line that secretes a human IgM reacting with G_{D3} and, to a lesser extent, G_{D2}, disialoparagloboside, G_{D1b}, G_{M3}, and G_{T1b} (10). B16/T28 is a mouse B78 melanoma line that has been stably transfected with human G_{D3} synthase gene (11), resulting in high levels of G_{D3} expression. This cell line was a

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[4] Abbreviation used in this paper: CDR, complementarity-determining regions.

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gift from Drs. Kenneth Lloyd and Koichi Furukawa. Sp2/0, a mouse myeloma cell, was purchased from American Type Culture Collection (Rockville, MD).

Site-directed mutagenesis

ChR24-pdHL2.4, an expression vector that encodes for the coexpression of R24 light and heavy chain variable region genes with the constant regions of human IgG1κ (8) was provided by Dr. Alan Jarvis (Repligen Corp., Cambridge, MA). Site-directed mutagenesis was performed in one of two ways. In the first method, a Muta-Gene Phagemid In Vitro Mutagenesis kit (New England BioLabs, Beverly, MA) was used. DNA encoding the V_H region of chR24 was isolated from the chR24-pdHL2.4 expression vector as a *Hin*dIII/*Xho*I restriction fragment, ligated into pBluescript II (Stratagene, La Jolla, CA), and cloned in *Escherichia coli*. Single stranded DNA was obtained, and in vitro mutagenesis was performed using a set of oligomers designed to introduce amino acid substitutions at specific codons within CDR2 of V_H. Each mutagenized V_H was sequenced by the dideoxy chain termination method using Sequenase version 2.0 DNA (U.S. Biochemical Corp. Cleveland, OH). The mutagenized V_H was isolated by *Hin*dIII/*Xho*I digestion and religated into the pdHL2.4 expression vector. The V_H insert was resequenced to confirm the presence of the appropriate V_H mutation.

The second method of site-directed mutagenesis used the overlap extension PCR method (12). For the primary PCR reactions, complimentary strands of chR24 V_H DNA were used as template. Two separate PCR products were generated with the desired mutations incorporated into overlapping 5′ and 3′ ends, respectively, by appropriately designed primers. These primers were also designed to introduce a *Hin*dIII or *Xho*I site, respectively, into the opposite end of each PCR product. The two overlapping PCR products served as the template for the second PCR reaction to generate the full-length V_H segment containing the desired mutations. All PCR reactions were performed in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) using the following program: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. The mutagenized V_H was inserted into the pCRII vector (Invitrogen, San Diego, CA) and sequenced to confirm the mutation. A *Hin*dIII/*Xho*I restriction fragment containing the mutagenized V_H was then subcloned into the chR24-pdHL2.4 vector. After each subcloning, the mutagenized V_H was resequenced to confirm that no further mutational changes had occurred.

Cell culture and transfection

Transfection of the various chR24-pdHL2.4 constructs was accomplished by protoplast fusion with Sp2/0.Ag14 myeloma cells. Protoplasts were prepared from cultures of recombinant *E. coli* by pelleting the cells and resuspending them in 2.5 ml of cold 20% sucrose in 50 mM Tris-HCl, pH 8.0, followed by the addition of 0.5 ml of lysozyme (5 mg/ml) in 250 mM Tris-HCl, pH 8.0, and 1 ml of 250 mM EDTA, pH 8.0. After incubation on ice for 5 min, an additional 1 ml of Tris buffer was added, and the mixture was incubated at 37°C for 10 min. After formation of protoplasts, 20 ml of 10% sucrose in DMEM were added, and 15 min later, the protoplast suspension was used. For each fusion, 5×10^6 Sp2/0 cells were fused with 2×10^9 protoplasts in 1 ml of 50% Polyethylene glycol 1450 (Sigma Chemical Co., St. Louis, MO) for 90 s at room temperature. The fused cells were washed three times with DMEM containing 1% FCS, 100 U/ml of penicillin, and 100 U/ml of streptomycin, and then were resuspended in 10 ml of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1% nonessential amino acids, 100 U/ml of penicillin, 100 U/ml of streptomycin, and 100 μg/ml of kanamycin. The cells were then plated at 100 μl/well in 96-well plates and cultured at 37°C in 5% CO_2. After 24 h, an additional 100 μl of RPMI 1640 containing 100 nM methotrexate was added to prevent the growth of nonfused Sp2/0 cells. The culture supernatants were screened for secretion of human IgG using a capture ELISA in which supernatants were added to 96-well plates coated with goat anti-human IgG capture Abs (1 μg/ml). After washing, bound human IgG was detected using an alkaline phosphatase-conjugated goat anti-human IgG second Ab. The plates were developed using the method described below for the homophilic binding assay. Wells containing cells secreting human IgG were expanded for large scale Ab production.

mAb were purified from culture supernatants by protein G affinity chromatography (Pharmacia, Piscataway, NJ). The protein concentration was determined by the colorimetric Bradford protein assay (Bio-Rad, Richmond, CA) using IgG as a protein standard.

Sequencing V_H regions of mAb

mRNA was obtained from each hybridoma (FastTrack, Invitrogen), and first strand cDNA synthesis was accomplished (cDNA Cycle Kit, Invitrogen) and used as a template for PCR amplification of each V_H region. For mAb 11A, the 5′ primer (5′-GACGTGAAGCTGGTGGA-3′) was designed based on the N-terminal amino acid sequence of 11A, and the reverse primer (5′-GGAAGACATTTGGGA-3′) was based on conserved sequences within IgM CH1 region. A second PCR reaction was performed using a nested forward primer (5′-GGGAGGCTTAGTGAA-3′). PCR reactions for mAb R24, C5, K9, and HJM1 used Ig-Prime System primer sets purchased from Novagen (Madison, WI). Amplified V_H DNA was ligated into pCRII cloning vector, cloned in *E. coli*, and sequenced. Each mAb was subjected to N-terminal amino acid sequencing to confirm that the translated amino acid sequences obtained were correct. For mAb MB3.6, a cDNA library was constructed, and full-length clones were selected using a probe to the C_H region. The cDNA was cloned into an expression vector and sequenced. The deduced amino acid sequence was confirmed by partial direct sequencing of the mAb.

Serologic analyses

For the homophilic binding assays, 96-well plates were coated with decreasing concentrations of target mAb diluted in 0.05 M Na borate, pH 8.6, overnight at 4°C. mAb R24 (10 μg/ml) was added to each well for 1 h. After washing, bound R24 was detected with an alkaline phosphatase-conjugated goat anti-mouse IgG3 second Ab. The plate was developed by adding *p*-nitrophenyl phosphate in a diethanolamine buffer. Absorbance at 405 nm was determined using a plate reader. Binding of mAb to purified G_{D3} was measured by ELISA as previously described (13).

Immuno-TLC was performed with purified gangliosides as well as mixed melanoma ganglioside preparations, essentially as previously described (14), except that bound mAb was visualized using a peroxidase-conjugated goat anti-human IgG second Ab.

Flow cytometry

B16/T28 cells were incubated on ice for 1 h in RPMI 1640 supplemented with 10% FCS containing 100 μg/ml of mAb. Cells were washed three times with PBS/1% BSA/0.05% Tween-20, and the pellet was resuspended in RPMI 1640 containing FITC-conjugated anti-human IgG (Tago, Burlington, CA) at a 1/20 dilution. After 1-h incubation, the cells were again washed, resuspended in 0.5% paraformaldehyde, and analyzed using an EPICS-Profile II fluorescence-activated flow cytometer (Coulter Corp., Miami, FL).

X-ray crystallography and computer modeling of R24 Fab fragments

The three-dimensional structure of the R24 Fab was determined by x-ray crystallography, the details of which will be published separately. A conservative approach was taken in modeling G_{D3} into the observed binding pocket of R24. No side chains were permitted to move more than 0.5A, and no main chain atoms were moved. Modeling was conducted using SETOR (15). The model of the two sialic acid residues of G_{D3} was generated from the program GEGOP (unpublished). Docking was performed by allowing conformational freedom to carbohydrate side chains. The model of G_{D3}-R24 that resulted contains several hydrogen bonds between the carbohydrate and Fab, and possesses a high degree of complementarity.

Results

Sequence analysis of variable regions of mAb that recognize G_{D3} ganglioside

We have previously shown that the V_H regions of anti-G_{D3} mouse mAb R24, C5, K9, 11A, and MB3.6 all express the Id_{HOM} for homophilic binding (5). HJM1, a human anti-G_{D3} IgM, does not express this idiotope, but can bind to Id_{HOM} expressed on murine anti-G_{D3} mAb (data not shown). To characterize and map Id_{HOM}, V_H regions of these anti-G_{D3} mAb were sequenced and analyzed (Fig. 1). The first observation was that four of five mouse mAb directed against G_{D3} (R24, C5, 11A, and MB3.6) use a V_H segment from the most J_H-proximal gene family, V7183 (16), and all five use a joining region from the J2 gene family (17). Identification of the diversity segment was possible only for R24 and C5, and both use a member of the VQ52 family (18). The sequence of an additional mouse anti-G_{D3} mAb, designated KM871, has been reported previously (19), and we noted that this mAb also uses the V7183 and J2 gene families to generate V_H. Thus, five of six sequenced mouse mAb against G_{D3} use the same V and J gene families to form V_H regions. These observations suggest that the

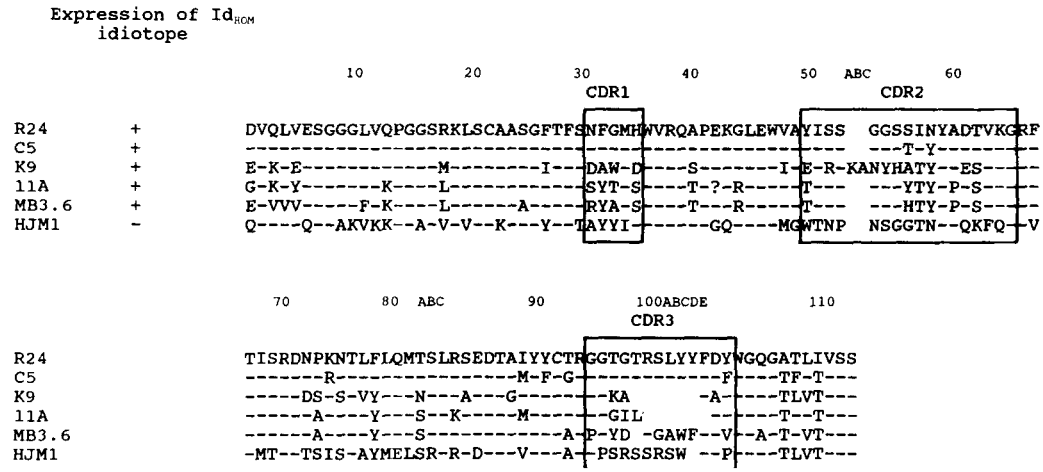

FIGURE 1. Derived V_H amino acid sequences of mAb directed against G_{D3} ganglioside. R24, C5, K9, 11A, and MB3.6 are mouse anti-G_{D3} mAb and express the Id_{HOM} idiotope. HJM1 is a human IgM mAb directed against G_{D3} and does not express this idiotope. Numbering of amino acids is according to the method of Kabat (20). CDRs are outlined.

murine immune response to G_{D3} is relatively stereotypic and that to produce high avidity Abs against G_{D3}, mouse B lymphocytes use a restricted set of VDJ gene families to form the V_H region.

Homophilic binding by mouse anti-G_{D3} mAb is specific for anti-G_{D3} mAb, since they do not bind to mAb specific for other Ags. This observation led us to hypothesize that the Id_{HOM} is expressed within a V_H CDR. A comparison of the V_H CDR amino acid sequences among the mAb that express Id_{HOM} indicated that the CDR1 and CDR3 sequences were highly variable and suggested no candidate idiotopes. The CDR2 sequence of R24, however, contains an eight-amino acid palindromic sequence starting at amino acid 51, of which the first six amino acids are conserved in mAb C5, 11A, MB3.6, and KM871 (19). In addition, it was noted that homophilic binding and expression of Id_{HOM} were associated with serine at position 52a (ser52a) and an acidic amino acid at position 61. Amino acids 62 to 65 of CDR2 (TVKG) were also conserved among these mAb, but since these residues are highly conserved among many mAb of various specificities (20), they were considered unlikely to define Id_{HOM}. These observations led us to test the role of ser52a and an acidic amino acid at position 61 in 1) expression of the Id_{HOM}, and 2) binding to G_{D3} ganglioside. We used a mouse-human chimeric form of the anti-G_{D3} mAb R24 (chR24) for these experiments, since the availability of the chR24-pdHL2.4 made it possible to carry out site-directed mutagenesis.

Effects of chR24 CDR2 V_H substitutions on Id_{HOM}

A panel of chR24 mutants with a single or double amino acid substitution at position 52a and/or 61 in the V_H CDR2 region were constructed and tested for the expression of Id_{HOM}. We found that Id_{HOM} was absolutely dependent on ser52a and was destroyed by any substitution at this position (Table I and Fig. 2). Remarkably, even a serine→threonine substitution (represented by variant LT52), in which a hydrogen is replaced with a methyl group, resulted in the complete loss of Id_{HOM} expression; however, substitutions at position 61 were less critical. An aspartic acid→glutamine substitution had no effect on Id_{HOM} expression, whereas an aspartic acid→histidine only partially destroyed Id_{HOM}, decreasing the ability of R24 to bind to Id_{HOM} by 10-fold. These results, summarized in Table I, were interpreted to demonstrate that the hydroxyl group of ser52a is critical for formation of Id_{HOM} and that there are stringent steric constraints at this position such that addition of a methyl group to the side chain at position 52a prevents binding to this idiotope.

Table I. *Effects of CDR2 substitutions at positions 52A and 61 in chR24 on expression of homophilic binding epitope and binding to G_{D3}*

ChR24 Variant	Amino Acid 52A	Amino Acid 61	Expression of Id_{HOM} Idiotope	Binding to G_{D3} by ELISA
R24[a]	S	D	+	+
LT52	T	D	−	+
R52	R	D	−	−
P52	P	D	−	−
D52	D	D	−	−
Y52	Y	D	−	−
Q61	S	Q	+	+
H61	S	H	+	+
R52E61	R	E	−	−
P52Q61	P	Q	−	−

[a] R24 represents the native form of chR24 with no amino acid substitutions.

Effects of CDR2 V_H substitutions on chR24 binding to G_{D3} ganglioside

The effect of V_H CDR2 substitutions on binding to G_{D3} was measured by both ELISA and flow cytometry. Substitutions at position 52a generally resulted in complete loss of binding to G_{D3} as measured by ELISA (Table I), suggesting that this residue is involved in the G_{D3} binding site. However, a serine→threonine substitution at position 52a, represented by variant LT52, evidently maintained the G_{D3} binding site (Fig. 3) and did not alter the specificity of the Ab as assessed by immuno-TLC against a mixture of melanoma gangliosides (data not shown). Results from experiments with variants Q61 and H61 demonstrate that substitutions at position 61 had no apparent effect on binding to G_{D3} (Fig. 3), indicating that this residue is not critical for either binding to G_{D3} or expression of Id_{HOM}.

Effect of homophilic binding on G_{D3} avidity

The observations that a serine→threonine substitution at position 52a resulted in the loss of Id_{HOM} but preserved the G_{D3} binding site suggested that Id_{HOM} is near, but not equivalent to, the G_{D3}

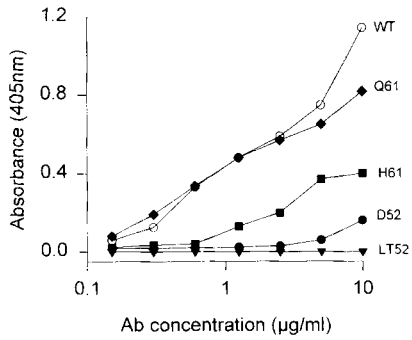

FIGURE 2. Expression of Id_{HOM} by chR24 and selected chR24 variants. ChR24, labeled WT in the figure for wild-type or variants Q61, H61, D52, and LT52 were coated onto ELISA plates. After blocking, decreasing concentrations of mouse R24 were added, and bound mouse R24 was detected using an alkaline phosphatase-conjugated goat anti-mouse IgG3 second Ab.

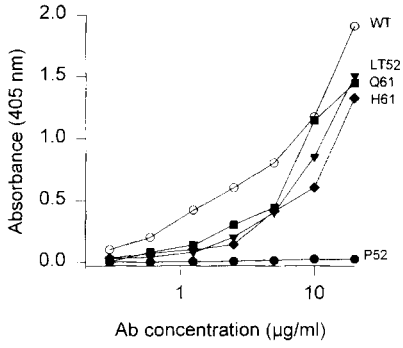

FIGURE 3. Binding of chR24 and selected chR24 variants to G_{D3} ganglioside as measured by ELISA. ChR24, labeled WT in the figure for wild-type, or variants H61, Q61, P52, and LT52 were tested for binding to G_{D3}-coated ELISA plates as described in *Materials and Methods*.

binding site. We were interested to determine how this substitution affected binding to cell surface G_{D3}. Substitutions at position 61 did not significantly affect binding to cell surface G_{D3} as measured by flow cytometry. On the other hand, LT52 demonstrated avidity for cell surface G_{D3} that was almost 2 orders of magnitude lower than that of native chR24, as measured by peak immunofluorescence (Fig. 4). This result contrasts with the ELISA results in which LT52 showed binding to G_{D3} equivalent to that of native R24 (Fig. 3). This disparity is explained by the fact that there are additional constraints in mAb binding to cell surface G_{D3} (discussed below). We interpret these results as demonstrating that LT52 has decreased avidity for G_{D3} compared with native chR24, but this was not evident by ELISA due to the high sensitivity of this assay.

These results demonstrate that despite an intact G_{D3} binding site, homophilic binding is required for high avidity binding to cell surface G_{D3}. An alternative explanation is that the serine→threonine substitution at position 52a not only destroyed Id_{HOM}, but also sufficiently altered the G_{D3} binding site to result in decreased avidity for G_{D3}. To determine the relative position of ser52a with respect to the G_{D3} binding site, the structure of the Fab from R24 was determined by x-ray crystallography, which permitted us to model the G_{D3} binding site of R24.

Computer modeling of the G_{D3} binding site and Id_{HOM}

The x-ray crystal structure of the Fab from R24 clearly showed a binding pocket into which the terminal sialic acid residue of G_{D3} could be docked (Fig. 5*A*). This model shows that histidine 35, tyrosine 50, and tyrosine 100c can participate in forming hydrogen bonds with oxygen molecules of G_{D3} and is consistent with previous R24 binding studies that showed that the terminal sialic acid of G_{D3} was critical for R24 binding (21). Ser52a is situated at the mouth of the G_{D3} binding pocket and does not interact with the terminal sialic acid residue of G_{D3}, although it is positioned to form hydrogen bonds with the second sialic acid residue. A threonine in place of serine at position 52a appears to alter the outer surface of this binding pocket while still allowing G_{D3} to bind (Fig. 5*B*). Other amino acid substitutions at position 52a, such as aspartic acid (Fig. 5*C*), would be expected to deform significantly the G_{D3} binding pocket, thus interfering with its ability to bind G_{D3}. This is consistent with the observation that substitution of any other amino acid, besides threonine, at position 52a resulted in the complete loss of avidity for G_{D3} (Table I).

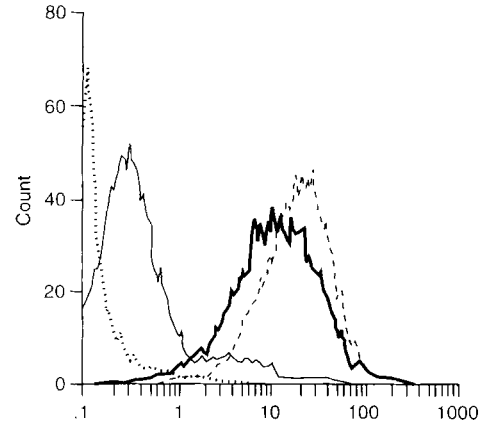

FIGURE 4. Binding of chR24 and chR24 variants to cell surface G_{D3}. Binding of chR24 (dashed line), Q61 (bold line), and LT52 (thin line) to the G_{D3}^+ melanoma cell line B16/T28 was measured by flow cytometry. Background binding (dotted line) was assessed by incubating B16/T28 with second Ab (FITC-conjugated goat anti-human IgG) in the absence of primary mAb. The *x*-axis represents relative fluorescence.

This model of the G_{D3} binding pocket is also consistent with the hypothesis that the decreased binding of LT52 to cell surface G_{D3} is not due to an altered G_{D3} binding site. The more likely explanation is that the intrinsic avidity of the G_{D3} binding pocket for G_{D3} is relatively low in the absence of homophilic binding and that homophilic binding increases the apparent avidity through enhanced multivalent binding. We conclude that homophilic binding is required for high avidity binding to cell surface G_{D3}.

Discussion

We reported previously that homophilic binding is a general characteristic of mouse anti-G_{D3} mAb and proposed a model in which the G_{D3} binding site of soluble mAb bound specifically to a V_H idiotope (now termed Id_{HOM}) on immobilized mAb (5). In the current report, studies with chR24 variants and computer modeling of the G_{D3} binding site provide further support for this model. Id_{HOM} absolutely requires a serine at position 52a, since any substitution at this position destroyed the idiotope. Although Id_{HOM} was not required for binding to G_{D3} by ELISA, most substitutions at position 52a destroyed both Id_{HOM} and the G_{D3} binding site. This implies that Id_{HOM}, as defined by ser52a, is near but not in the G_{D3} binding site. This was confirmed by the x-ray crystal structure of R24 Fab fragment, which revealed that the G_{D3} binding site is a pocket and predicted that amino acids within the pocket from V_H CDR1 (His35), CDR2 (Tyr50), and CDR3 (Tyr100c) form

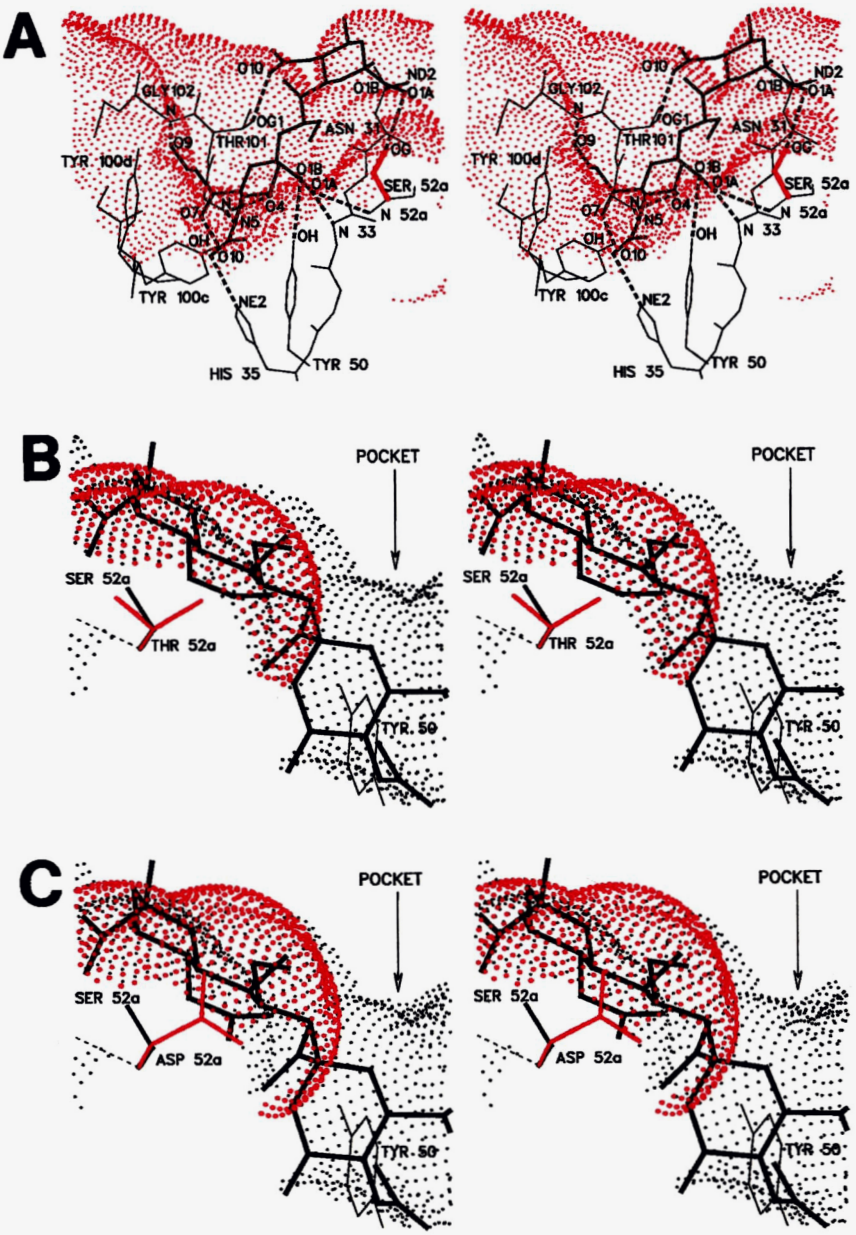

FIGURE 5. Molecular model of G_{D3} binding to R24 and R24 variants at residue 52a based on the x-ray crystal structure of the unliganded R24 Fab fragment. *A*, Stereoview of the modelled fit of the two terminal sialic acid residues of G_{D3} (thick lines) docked into the binding pocket observed on the surface of R24. Histidine 35, tyrosine 50, and tyrosine 100c are positioned to form hydrogen bonds (dotted lines) with oxygen molecules on G_{D3}. Serine 52a contacts G_{D3} and is positioned to hydrogen bond with the second sialic acid residue of G_{D3}. *B*, A close-up stereoview of the environment surrounding serine 52a showing the minimal change in molecular surface and orientation of the hydroxyl group that would occur if the native serine (black) was replaced with threonine (red). *C*, A close-up stereoview of the change in molecular surface if aspartic acid (red) is substituted for serine 52a (black). Unlike substitution by threonine (*B*), this substitution causes a large distortion in the Ag binding surface and displaces the hydrogen-binding moiety to an extent that it can no longer form the original hydrogen-bonding pattern. Views *B* and *C* are rotated approximately 180 degrees about the vertical axis compared with view *A*.

H-bonds with G_{D3}. Ser52a is at the mouth of the pocket which, as predicted, maps Id_{HOM} near the G_{D3} binding site, but separate from it. Modeling studies revealed that substitutions at position 52a, other than threonine, would be expected to significantly alter the G_{D3} binding pocket. Thus, both immunologic and x-ray crystallographic studies support the idea that Id_{HOM} is a V_H idiotope situated adjacent to the G_{D3} binding site.

Provided that the G_{D3} binding site of soluble R24 binds to Id_{HOM}, it is tempting to hypothesize that Id_{HOM} mimics G_{D3} ganglioside. In this regard, it has been proposed that a subset of Abs carry an internal image of their own Ag and that these Abs play a key role in the regulation of the humoral immune response through an Id network (22). Alternatively, it is possible that Id_{HOM} represents a binding site for an alternative ligand yet to be identified. In support of this possibility is the finding that V_H epitopes outside the traditional antigen binding site of other Ab molecules bind members of the Ig supergene family such as CD4 (3) and NCAM (4). In addition, binding sites for superantigens, such as staphylococcal protein A, have been mapped to framework and CDR2 regions of Ig and may play an important role in B cell regulation (1, 23). Thus, we do not rule out the possibility that Id_{HOM} serves as a binding site for other ligands. Although an alternative ligand that binds to Id_{HOM} has not been identified, we note that Id_{HOM} has significant homology with the third extracellular region of adenosine receptor A_1 (24), a region that to date has no defined function.

The binding characteristics of the LT52 variant of chR24 demonstrate that homophilic binding is not required for binding to G_{D3} measured by ELISA, but is required for high avidity binding to G_{D3} on the cell surface. This may be due to the fact that on an ELISA plate, G_{D3} is immobilized and at high density on a two-dimensional surface, conditions that decrease the degrees of freedom and allow low avidity interactions. In contrast, on the cell surface, G_{D3} is mobile, and its accessibility to mAb may be hindered by membrane glycoproteins that are generally 10 to 100 times larger than G_{D3}. Thus, homophilic binding represents a mechanism for providing high avidity binding to cell surface G_{D3},

which is crucial for complement-directed lysis and Ab-dependent cellular cytotoxicity, effector functions that anti-G_{D3} mAb mediate efficiently (13). This mechanism of high avidity binding could be especially important for Abs such as R24 that recognize nonprotein (so called T cell-independent) Ags. Somatic mutation, the classical mechanism for evolving a higher avidity Ab during the generation of a humoral immune response, requires T cell help that is not available in the case of a nonprotein Ag such as G_{D3}. However, if the B cell uses a V_H segment that contains a homophilic binding idiotope, it may result in the formation of higher avidity Abs without V region maturation requiring T cell help.

This model predicts that high avidity mAb against other T-independent Ags may demonstrate homophilic binding. Indeed, V_H-directed self-binding has been reported in the anti-phosphoryl choline mAb T15 and M603, in which an epitope within the V_H CDR2 region was implicated by the finding that CDR2-derived peptides could inhibit self-binding (25). A second example is mAb 26–10 directed against the glycoside digoxin. While this mAb was not tested directly for homophilic binding, a serine residue at position 52 in V_H CDR2 was found to be critical for high avidity binding to digoxin, even though ser52 did not contact Ag by x-ray crystallographic analysis (26). This finding raises the possibility that this ser52, like the ser52a of anti-G_{D3} mAb, provides an idiotope for self-binding that results in higher avidity binding to Ag. These two examples of anti-phosphoryl choline and anti-digoxin mAb contain elements in common with our model of homophilic binding among anti-G_{D3} mAb and are consistent with the idea that a variety of mAb against nonprotein Ags can exhibit homophilic binding. There is evidence, however, that homophilic binding may also occur among Abs against protein Ags. Autoantibodies against thyroglobulin have been reported to express an idiotope that not only allows self-binding, but also functions as an alternative binding site for thyroperoxidase (27). Taken together, these observations suggest that the V_H repertoire may contain multiple mechanisms for generating self-binding idiotopes.

We observed that in five of six mouse anti-G_{D3} mAb sequenced, the V_H region was formed by using a V_H gene from the V7183 gene family. The mouse V_H locus consists of 11 V_H families, of which V7183, one of the smallest families, is the most J_H proximal. In mature mouse B cells, V_H families are selected in proportion to the relative size of the gene family, indicating that V_H gene family usage is largely random. This is indicated by the observation that the VJ558 family is the most heavily represented. In contrast, differentiating pre-B cells are biased toward using the most J_H-proximal gene families, such as the V7183 and VQ52 families (28). The bias of mouse anti-G_{D3} mAb toward J_H-proximal V_H gene families strongly suggests that the murine Ab response to G_{D3} is the result of selection in a pre-B cell compartment rather than in mature B cells.

The bias of mouse anti-G_{D3} mAb toward J_H-proximal V_H gene families contrasts with the murine Ab response against other glycolipid Ags. The V_H genes used in 46 mouse mAb against various ganglioside and sulfated glycolipid Ags other than G_{D3} were derived from the large V_H gene families, consistent with random V_H gene family selection rather than J_H-proximal families (29). This suggests that the murine humoral immune response against non-G_{D3} gangliosides is produced by a mature B cell population and is consistent with the observation that Abs against these non-G_{D3} glycolipid Ags, such as G_{M1}, can develop spontaneously in older mice (30). Thus, the murine Ab response to G_{D3} appears to be qualitatively different from the Ab response to other ganglioside Ags, in that Abs against other gangliosides are produced by mature B cells selecting V_H gene families at random.

There is evidence that early in human ontogeny, the V_H gene usage is also biased toward J_H-proximal gene families (31). Thus, it is tempting to hypothesize that the situation in humans mirrors that in the mouse; namely, that mature human B cells can generate Abs against various carbohydrate Ags other than G_{D3}, but that it is the pre-B cell population that has a V_H gene usage bias that favors the generation of high avidity Abs against G_{D3}. Consistent with this hypothesis is the observation that in adults, Abs against a variety of ganglioside Ags, including G_{M2}, G_{D2}, and G_{D1a}, can arise naturally or can be induced by immunization (32–38), while high avidity Abs against G_{D3} do not develop spontaneously and have been relatively difficult to induce with immunization (35, 39). In contrast with mouse mAb against G_{D3}, human anti-G_{D3} mAb HJM1 exhibits relatively low avidity G_{D3} binding, does not demonstrate homophilic binding, and cross-reacts with other gangliosides. Consistent with this difference is the observation that HJM1 does not use a J_H-proximal V_H gene family and is probably the product of a mature human B cell.

This model of V_H gene family usage suggests possible approaches to induce high avidity Abs against G_{D3} in patients. Circulating $CD5^+$ pre-B cells, which can be detected in adult peripheral blood, preferentially express J_H-proximal V_H genes (40). This pre-B cell compartment is known to require certain growth factors, such as IL-7 (41), and we speculate that strategies to expand and activate this subpopulation of B cells before immunization could result in high avidity Abs against G_{D3}.

In summary, we have 1) defined the structural basis for homophilic binding among mouse anti-G_{D3} mAb, 2) generated a model for G_{D3} binding to R24 from the structure of the unliganded Fab that is consistent with immunologic data, and 3) demonstrated that homophilic binding is required for high avidity binding to cell surface G_{D3}. These studies also suggest that the murine Ab response to G_{D3} results from activation of a pre-B cell compartment. This is in contrast to the Ab responses to other ganglioside Ags, which appear to rely primarily on mature B cells. If similar constraints apply to the anti-G_{D3} response in humans, strategies to activate pre-B cells that preferentially use J_H-proximal V_H gene families may be successful in inducing high avidity anti-G_{D3} Abs.

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