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Role of Heavy Chain Constant Domains in Antibody-Antigen Interaction

Apparent Specificity Differences among Streptococcal IgG Antibodies Expressing Identical Variable Domains[1,2]

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Abstract. In this report, we examine the influence of C_H domains on antibody specificity, in the context of variable epitope density on bacteria and synthetic glycoconjugates. Hybridomas secreting IgG1 and IgG2b mAb, specific for the *N*-acetyl-glucosamine (GlcNAc) residues of streptococcal group A carbohydrate, were previously generated from a hybridoma secreting a mouse IgG3 mAb. We show that these three mAb have identical H and L chain V domains, as determined by 1) cDNA sequencing, 2) binding to soluble Ag, and 3) binding to nine monoclonal anti-idiotopes. Nevertheless, the IgG3 mAb binds more effectively than the V region-identical IgG1 or IgG2b mAb to each of three strains of group A streptococci that display different amounts of terminal GlcNAc residues on their cell walls. The magnitude of the subclass-associated differential in binding varies with the target strain, and, whereas the IgG3 mAb binds best to the strain expressing an intermediate amount of GlcNAc, the IgG1 and IgG2b mAb and IgG3-derived $F(ab')_2$ fragments bind best to the strain expressing the highest amount of GlcNAc. The IgG3 mAb also binds better than the IgG1 and IgG2b mAb to solid-phase $GlcNAc_{50}$-BSA, but the IgG2b mAb binds best to otherwise identical conjugates with lower ratios of GlcNAc to BSA (20:1, 10:1, 5:1, and 1:1). These results suggest that epitope density can significantly influence the magnitude of IgG subclass-associated binding differences, and that structural differences in the C_H regions, particularly the C_H2 and C_H3 domains, can influence the apparent specificities of IgG molecules for multivalent Ag. *Journal of Immunology,* 1993, 150: 2231.

Antibody specificity generally is attributed to the ability of the V domains to discriminate among epitopes exhibiting subtle molecular differences. However, antigenic particles, including bacteria, viruses, and parasites, can differ from one another and from host components not only with respect to the details of epitope structure, but also in terms of epitope spacing. Several investigators have shown that epitope density can markedly affect the binding of antibodies to solid-phase Ag (1, 2). Based on electron microscopic studies of the distributions of bound antibodies, it has been claimed that interactions between bound antibodies (lateral interactions) can influence the amount of antibody bound, and that epitope density can influence the occurrence of such interantibody contacts (1). However, the implications of such effects for the specificity of antibody binding to antigenic targets of differing epitope densities, especially where the antibodies are of different IgG subclasses, have not been systematically explored. In this report, we analyze IgG subclass-related

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differences in antibody binding and apparent specificity in the context of variable epitope density.

We have selected IgG1 (HGAC 39.G1)[5] and IgG2b (HGAC 39.G2b) switch variant mAb from a mouse hybridoma secreting an IgG3 mAb (HGAC 39.G3) specific for (GlcNAc) residues of GAC. The IgG3 mAb was found to bind more strongly to group A intermediate streptococci than the V region-identical IgG1 and IgG2b mAb (3). In this report, we confirm that the switch variant IgG1 and IgG2b mAb express V domains identical to those of the IgG3 mAb, and we extend the previous results by comparing the interactions of all three mAb with three streptococcal strains displaying different amounts of cell wall GlcNAc residues and with GlcNAc-BSA conjugates of varying substitution ratios. We find that the IgG3 mAb binds more effectively than the V region-identical IgG1 or IgG2b mAb to each of the three bacterial strains. However, the magnitude of this IgG subclass-associated binding difference varies with the target strain. Furthermore, although the IgG1 and IgG2b mAb and $F(ab')_2$ fragments derived from the IgG3 mAb bind best to the strain expressing the highest average density of GlcNAc, the IgG3 mAb binds best to the streptococcal strain expressing an intermediate amount of GlcNAc. In experiments using a set of GlcNAc-BSA conjugates, the IgG3 mAb binds better than the IgG2b and IgG1 mAb to the highest epitope density conjugate (50:1). However, the IgG2b mAb binds better than the IgG3 and IgG1 mAb to the conjugates with lower epitope densities (20:1, 10:1, 5:1, 1:1). These results support the premise that the C_H domains, in addition to the V domains, can influence apparent antibody specificity for multivalent Ag that differ with respect to epitope density.

Materials and Methods

mAb

The mouse hybridoma HGAC 39.G3 (previously referred to as HGAC 39) secretes an IgG3, κ mAb with specificity for the GlcNAc residues of the cell wall polysaccharide from group A streptococci (4). Hybridomas secreting IgG subclass switch variants were selected from cells secreting HGAC 39.G3, using polyclonal goat antibody specific, respectively, for the mouse γ1 or γ2b heavy chain constant regions (FisherBiotech, Pittsburgh, PA), as described (3). The change of subclass was confirmed using an additional subclass-specific polyclonal antibody, distinct from the antibody used for selection, and rat mAb specific for mouse IgG1 (Zymed Laboratories, South San Francisco, CA) or IgG2b (Pharmingen, San Diego, CA), respectively. The hybridomas secreting the IgG1 (HGAC 39.G1) or IgG2b (HGAC 39.G2b) subclass variant mAb were cloned by limiting dilution once at 0.3 cells/well and two times at 0.1 or 0.2 cells/well on Terasaki trays (Robbins Scientific, Sunnyvale, CA) (5).

The mAb were purified from hybridoma supernatant by affinity chromatography using GlcNAc conjugated to agarose beads (Sigma, St. Louis, MO). Each column of GlcNAc-agarose beads was used with only one type of mAb preparation to prevent cross-contamination. The concentration of the mAb was determined by absorbance at 280 nm in the absence of sodium azide [$E^{0.1\%}_{280\,nm}$ = 1.36 OD μ/ml (6)]. The functional concentrations of the mAb preparations were validated using an ELISA to determine reactivity with κ-specific goat antibody (see below). Sodium azide was added at a final concentration of 0.02% and the antibody preparations were stored at 4°C. Serum-free supernatants containing secreted mAb were generated from hybridoma cells that had been washed twice with serum-free medium prior to culture under serum-free conditions so as to reduce potential contamination from horse serum proteins. The myeloma proteins (Sigma) FLOPC 21 (IgG3), MOPC 21 (IgG1), and MOPC 141 (IgG2b) were used as nonspecific IgG subclass-matched control antibodies.

$F(ab')_2$ fragments were generated from affinity-purified HGAC 39.G3 after digestion with immobilized pepsin. A 2.5-ml aliquot of HGAC 39.G3 (mouse mAb IgG3, κ) at 1.9 mg/ml was digested with 190 mg pepsin-agarose (Sigma; 0.22 U/mg solid, 130 U/mg dry agarose) in 100 mM sodium acetate, pH 4.5, for 45 min at 37°C. The results of the digestion were analyzed using a Coomassie R 350 stain of nonreducing SDS-PAGE gels performed at 3 W constant power, using a 10% to 15% polyacrylamide gradient (Pharmacia LKB Biotechnology, Piscataway, NJ; PhastSystem). These digestion conditions were chosen to minimize possible enzyme-mediated damage to the antibody variable domains. The $F(ab')_2$ fragments were passed over Protein A coupled to agarose (Pierce, Rockford, IL) to reduce contamination by intact IgG3 mAb. These fragments were further purified by affinity chromatography using GlcNAc-agarose.

cDNA sequencing

RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (7) from hybridoma cells secreting HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b. Poly (A^+) RNA was purified using an oligo-(dT) cellulose column (Collaborative Research, Bedford, MA) (8). The heavy and light chain mRNAs were reverse transcribed with 22 ng of the heavy chain constant region primers [γ3, $^{5'}$AGGGACCAAGGGATAGAC$^{3'}$; γ1 and γ2b, $^{5'}$GGGGCCAGTGGATAGAC$^{3'}$; (9)] or the light chain

[5] Abbreviations used in this paper: HGAC 39 mAb, mouse IgG3 (HGAC 39.G3), IgG1 (HGAC 39.G1), and IgG2b (HGAC 39.G2b) mAb specific for GAC and expressing the same V domains; HPCG, 11.G3 mA6, mouse IgG3 mAb specific for phosphorycholine; GAC; Group A carbohydrate; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNAc_x$-BSA, GlcNAc conjugated to bovine serum albumin at a ratio of × moles of GlcNAc per mole of BSA; GAV, group A vaccine; GAiV, group A intermediate vaccine; GAvV, group A variant vaccine; Gal, galactose; [UDP]-Gal, uridine 5′-diphosphogalactose; EcorA, *Erythrina corallodendron* agglutinin; PCR, polymerase chain reaction.

constant region primer [κ, 5′TGGATGGTGGGAAGATG$^{3'}$; (10)] with 28 U AMV reverse transcriptase (USB, Cleveland, OH). A 1/100 dilution of the newly synthesized cDNA was amplified by PCR (1 min 94°C; 1 min 94°C, 1 min 55°C, 1 min 72°C, for 25 to 30 cycles; 1 min 72°C) using 10 μM κ light chain leader sequence primer [$^{5'}$CTGCTTGTGCTCTGGATC$^{3'}$ (11)] or heavy chain leader sequence primer [$^{5'}$ACTTGAGACTGAGCTGTG$^{3'}$ (12)] with 10 μM of one of the appropriate constant region primers. The amplified DNA was phenol-extracted, agarose gel purified, and blunt-end ligated into BlueScript (Stratagene, La Jolka, CA), which was then used to transform competent bacteria. White colonies of bacteria were expanded, and plasmid DNA was purified after alkaline lysis. Restriction enzyme analysis was performed to confirm the presence of inserted V region DNA and the insert DNA was sequenced by the Sanger method using Sequenase (USB), ^{35}S dATP, and primers flanking the BlueScript multiple cloning site. The products of the sequencing reaction were electrophoresed using an electrolyte gradient (13) on a 6% acrylamide gel containing 8 M urea. The bands were visualized by autoradiography using X-OMAT-AR (Kodak XAR 5, Rochester NY) film.

Biotin-conjugation of antibody

Antibody was conjugated at a ratio of 25:1 (biotin:mAb) with *N*-hydroxy-succinimide-X-biotin (Biotin-X-NHS; Calbiochem, San Diego, CA) using standard methods (14). The concentration of biotin-conjugated mAb was determined by absorbance at OD_{280}.

Streptococci

The group A intermediate and group A variant strains were originally derived from group A streptococci (*Streptococcus pyogenes*) by mouse passage (15). The bacteria were used in the assays as heat-killed and pepsin-treated preparations (GAV, GAiV, GAvV). Cultures of bacteria were grown from frozen stocks in Todd-Hewitt broth at 37°C, heat-inactivated at 56°C for 180 min and digested with pepsin [per liter of bacterial culture: 12.5 mg pepsin (from porcine stomach, 3900 U/mg protein, Sigma) in 5.5 ml of 0.15 M NaCl and 0.75 ml 1 N HCl, passed through 0.2 μm filter]. The pepsin was removed by thoroughly washing the bacteria at least four times with PBS.

Galactosylation of bacteria

GAV, GAiV, or GAvV were diluted to 2.4 OD_{650} absorbance U/tube and washed twice with 50 mM Tris buffer (pH 8 at 37°C). The bacterial vaccines were then suspended in 50 μl of galactosylation reaction mixture. The reaction mixture was made up on ice as follows: 2 U/ml of bovine 4-galactosyl transferase (9.5 U/mg protein, Sigma), 10 mM $MnCl_2$ (Aldrich, Milwaukee, WI; freshly diluted in H_2O), 2.5 mM 5′ AMP (freshly diluted in H_2O; Sigma), and 0 to 10 mM (saturating conditions) of the substrate uridine 5′-diphosphogalactose sodium salt ([UDP]-Gal; Sigma), diluted in 50 mM Tris buffer (pH 8 at 37°C) (16). In the presence of manganese ions, galactosyl transferase catalyzes the transfer of galactose (Gal) from [UDP]-Gal to nonreducing GlcNAc residues to give *N*-acetyllactosamine (17). The galactosylation reaction proceeded for 60 min while the bacteria rotated at 37°C. After washing with 1% BSA-PBS, the bacteria were diluted to 0.84 OD_{650} absorbance U/tube. The 80 μg/ml of biotin-conjugated HGAC 39.G3 or 100 μg/ml of biotin-conjugated lectin ECorA (approximately 5 mol biotin/mol protein; Sigma) was incubated with the treated bacteria. ECorA recognizes D-Gal-(1–4) D-GlcNAc (18). Unbound biotin conjugates were removed by washing and bound label was quantified after addition of a 1/50 dilution in 1% BSA-PBS of FITC-coupled avidin (Zymed Laboratories) using flow cytometry.

Synthetic glycoconjugates

$GlcNAc_{16}$-BSA was purchased from Sigma. In addition, a panel of GlcNAc-BSA conjugates with GlcNAc:BSA molar ratios of 50:1, 20:1, 10:1, 5:1, and 1:1 were synthesized using a two-step reaction. First, *p*-aminophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma) was activated by an equimolar amount of glutaric dialdehyde in 0.1 M Na-carbonate buffer at pH 9.0 for 30 min at 20°C. The activated glucopyranoside was then mixed with BSA in the same buffer and allowed to react for 1 h at 20°C. The product of this reaction was dialyzed against 0.05 M Tris-HCl buffer at pH 8.5 and then applied to a DEAE-Sephacryl (Pharmacia) column equilibrated with 0.05 M Tris-HCl buffer at pH 8.5. A step gradient of NaCl (0.005 M, 0.01 M, 0.02 M, 0.03 M, 0.05 M) in the same buffer was then used to elute the glycoconjugate. The concentration of conjugated hapten was determined by periodate assay (19).

Flow cytometry to measure antibody and lectin binding

The methods for the flow cytometry experiments are similar to those previously described (3). The 0.06 $O.D._{650}$ absorbance U/tube of GAV, GAiV, GAvV or $\sim 1.45 \times 10^8$ beads/tube were incubated with antibody or the biotin-conjugated ECorA overnight at 4°C, 24°C, or 37°C. The beads (Polysciences, Warrington, PA; 1 μ carboxylate polystyrene microparticles) were previously adsorbed with polyclonal goat antibody to mouse κ determinants (FisherBiotech) and blocked with 1% BSA-PBS. Unbound antibody or lectin was removed by washing with 1% BSA-PBS. The detection of bound antibody was made possible by the addition of FITC conjugated-polyclonal goat antibody specific for mouse κ light chains (FisherBiotech). The preparations were analyzed as reported (3).

ELISA to compare antibody binding to streptococci or GlcNAc-BSA

The methods have been described (3). Briefly, flexible 96-well polyvinyl chloride microtiter wells (Dynatech, Pittsburgh, PA) were coated by drying, at 24°C or 37°C, with comparable amounts (based on absorption at OD_{650}) of each bacterial vaccine (GAV, GAiV, GAvV). Alternatively, 5 μg/ml of GlcNAc-BSA in PBS was allowed to adsorb to the wells overnight, at 4°C. Antibody diluted in 1% BSA-PBS was incubated overnight with Ag, and bound antibody typically was detected after addition of alkaline phosphatase-conjugated polyclonal goat anti-mouse κ at 0.4 μg/ml. The color reaction was measured at 405 nm (absorbance units) after addition of *p*-nitrophenyl phosphate. Nonspecific binding of the labeled antibody to the solid-phase bacterial strains or GlcNAc-BSA was subtracted from the mean mAb binding value.

ELISA to measure antibody binding to anti-idiotopes

Hybridoma supernatants containing nine rat mAb anti-idiotypic antibodies were bound to the solid phase using 2 μg/ml of a mouse mAb specific for rat κ light chains. HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, and HPCG 11.G3 conjugated with biotin (via a *N*-hydroxysuccinyl ester) were assayed for binding to the anti-idiotypic mAb at 4°C. Binding of the biotin-conjugated mAb was detected using alkaline phosphatase conjugated to streptavidin (Zymed) diluted 1/1000 in 1% BSA-PBS. The relative specific activities of the biotin-conjugated mAb were determined by binding to solid-phase goat antibody to mouse κ light chains (FisherBiotech).

ELISA to compare the functional concentrations of mAb preparations

Microtiter plates were adsorbed with 1 μg/ml polyclonal goat antibody specific for mouse κ determinants (FisherBiotech). A comparison was made of the relative abilities of the mAb preparations, diluted in 1% BSA-PBS, to compete against a biotin-conjugated IgG1 κ mAb (5 or 10 ng/ml), for the binding to solid-phase goat antibody, overnight at either 24°C or 37°C. After washing with PBS to remove the unbound mAb, the bound biotin-conjugate was detected using 1/1000 dilution of alkaline-phosphatase-conjugated streptavidin (Zymed Laboratories).

RIA to measure binding of radiolabeled GlcNAc-BSA to mAb

In 96-well polystyrene microtiter plates, an excess of mouse mAb was immobilized by binding at 4°C to 2 μg/ml of adsorbed goat polyclonal antibody specific for mouse γ3, γ1, or γ2b H chains, respectively (FisherBiotech). Unbound mouse antibody was removed by washing with PBS, and the plates were blocked with 1% BSA-PBS or BLOTTO (5% non-fat dried milk in PBS). GlcNAc-BSA (approximately 16 mol GlcNAc/mol BSA), labeled by the chloramine T method (20) with ^{125}I (Amersham, Arlington Heights, IL), was titrated or added at a fixed concentration (20 μl/well) in the presence of monovalent hapten. The plates were incubated overnight at room temperature, unbound radiolabel was removed by washing, the wells were cut from the plates, and the amount of radiolabel bound to each well was determined with a gamma counter. The mean background binding of radiolabeled $GlcNAc_{16}$-BSA to the adsorbed goat antibody was subtracted from the mean of data points.

Results

Characterization of mAb V domains

The cDNA nucleotide sequences encoding the heavy and light chain V domains of the HGAC 39.G3 (IgG3), HGAC 39.G1 (IgG1), and HGAC 39.G2b (IgG2b) mAb were determined, as described in *Materials and Methods*. All three V_H and V_L domains, respectively, were shown to be encoded by identical nucleotide sequences (Fig. 1). Therefore, the V_H and V_L domains of the three mAb have identical deduced amino acid sequences (Fig. 1). Comparable concentrations of soluble GlcNAc inhibit 50% of the binding of radiolabeled $GlcNAc_{16}$-BSA to each of the three mAb, attached to the solid phase through previously adsorbed anti-heavy chain isotype antibodies (Fig. 2*a*), and the direct binding of radiolabeled GlcNAc-BSA to the immobilized HGAC 39 mAb is similar for all three mAb (Fig. 2*b*). GalNAc did not significantly interfere with the binding of iodinated GlcNAc-BSA to the solid-phase mAb. The results in Figure 3 demonstrate that nine of nine rat monoclonal anti-idiotopes, originally elicited by immunization with HGAC 39.G3 (22), bind comparably to biotin-conjugated preparations of all three mAb. These anti-idiotopes previously have been shown to bind over a large portion of the HGAC 39.G3 V module surface (23). No significant binding was seen to a control biotin-conjugated antibody (HPCG 11.G3) of irrelevant specificity. The specific activities of these biotinylated mAb preparations are similar for they bound comparably to solid-phase goat antibody with specificity for mouse κ constant domains (data not shown). Thus, binding of soluble $GlcNAc_{16}$-BSA (Fig. 2) and monoclonal anti-idiotopes (Fig. 3) to the IgG3, IgG1, and IgG2b mAb suggest that the V domains associated with these three mAb are similar in conformation. We conclude that HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b have identical V domains.

Binding of mAb to streptococci with different average epitope densities

Analysis of bacterial composition suggested that group A (parent), group A intermediate, and group A variant bear

A HEAVY CHAIN VARIABLE DOMAIN (and partial C_H1 domain in bold type face)

```
                Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln
HGAC 39.G3      GAA GTG AAG CTT GAG GAG TCT GGA GGA GGC TTG GTG CAA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Pro Gly Gly Ser Met Lys Leu Ser Cys Val Ala Ser Gly
HGAC 39.G3      CCT GGA GGA TCC ATG AAA CTC TCC TGT GTT GCC TCT GGA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Phe Thr Phe Ser Asn Tyr Trp Met Asp Trp Val Arg Gln
HGAC 39.G3      TTC ACT TTC AGT AAC TAC TGG ATG GAC TGG GTC CGC CAG
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Glu Ile Arg
HGAC 39.G3      TCT CCA GAG AAG GGA CTT GAG TGG GTT GCT GAA ATT AGA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Leu Lys Ser Asp Asn Phe Ala Thr His Tyr Ala Glu Ser
HGAC 39.G3      TTG AAA TCT GAT AAT TTT GCA ACA CAT TAT GCG GAG TCT
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys
HGAC 39.G3      GTG AAA GGG AGG TTC ACC ATC TCA AGA GAT GAT TCC AAA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Ser Ser VaL Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu
HGAC 39.G3      AGT AGT GTC TAC CTG CAA ATG AAC AAC TTA AGA GCT GAA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Asp Thr Gly Ile Tyr Tyr Cys Val Asp Leu Ser Trp Phe
HGAC 39.G3      GAC ACT GGC ATT TAT TAC TGT GTG GAC TTG AGC TGG TTT
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala
HGAC 39.G3      GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

HGAC 39.G3      GCT ACA ACA ACA GCC CCA TCT GTC TAT CCC TTG GTC
HGAC 39.G1      --C -A- --G --- C-- --- --- --- --- --A C-- -C-
HGAC 39.G2b     --C -A- --- --- C-- --- --A --- --- --A C-- -C-
```

B LIGHT CHAIN VARIABLE DOMAIN (and partial C_κ domain in bold type face)

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                Asp Ile Val Met Thr Gln Ala Ala Phe Ser Asn Pro Val
HGAC 39.G3      GAT ATT GTG ATG ACG CAG GCT GCC TTC TCC AAT CCA GTC
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Thr Leu Gly Thr Ser Ala Ser Ile Ser Cys Arg Ser Ser
HGAC 39.G3      ACT CTT GGA ACA TCA GCT TCC ATC TCC TGC AGG TCT AGT
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Lys Asn Leu Leu His Ser Asn Gly Ile Thr Phe Leu Tyr
HGAC 39.G3      AAG AAT CTC CTA CAT AGT AAT GGC ATC ACT TTT TTA TAT
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC39.G2b      --- --- --- --- --- --- --- --- --- --- --- --- ---

                Trp Tyr Leu Gln Arg Pro Gly Gln Ser Pro Gln Leu Leu
HGAC 39.G3      TGG TAT CTC CAG AGG CCA GGC CAG TCT CCT CAG CTC CTG
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Ile Tyr Arg Val Ser Asn Leu Ala Ser Gly Val Pro Asn
HGAC 39.G3      ATA TAT CGG GTG TCC AAT CTG GCC TCA GGA GTC CCA AAC
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Arg Phe Ser Gly Ser Glu Ser Gly Thr Asp Phe Thr Leu
HGAC 39.G3      AGG TTC AGT GGC AGT GAG TCA GGA ACT GAT TTC ACA CTG
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr
HGAC 39.G3      AGA ATC AGC AGA GTG GAG GCT GAG GAT GTG GGT GTT TAT
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Tyr Cys Ala Gln Leu Leu Glu Leu Pro Tyr Thr Phe Gly
HGAC 39.G3      TAC TGT GCT CAA CTG CTA GAA CTC CCG TAC ACG TTC GGA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

                Gly Gly Thr Lys Leu Glu Ile Lys
HGAC 39.G3      GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCA
HGAC 39.G1      --- --- --- --- --- --- --- --- --- --- --- --- ---
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- --- --- --- ---

HGAC 39.G3      CCA ACT GTA TCC ATC TTC CCA CCA TCC A
HGAC 39.G1      --- --- --- --- --- --- --- --- --- -
HGAC 39.G2b     --- --- --- --- --- --- --- --- --- -
```

FIGURE 1. DNA nucleotide sequence, using PCR-amplified cDNA obtained from reverse transcription of mRNA, of the *a*, heavy and *b*, light chain variable regions of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b. The deduced amino acid sequence is shown, and these sequences are in full agreement with a previously published sequence of HGAC 39.G3 (21).

differing amounts of GlcNAc (group A > group A intermediate >> group A variant) attached to the polyrhamnose backbone of the cell wall polysaccharide (15). We sought to verify these relationships using bovine milk galactosyl transferase to covalently add galactose residues to nonreducing GlcNAc termini, and the lectin, ECorA, to detect the terminal *N*-acetyl-lactosamine residues thereby created. Based on this assay (Fig. 4), GAiV expresses an intermediate amount of enzyme- and lectin-accessible GlcNAc residues, relative to group A and group A variant streptococcal vaccines (GAV and GAvV). The hierarchy of binding is independent of lectin concentration (data not shown). Divergence in the amount of cell wall GlcNAc was not explained by size variation among the streptococcal strains, as all three strains exhibited comparable dimensions by flow cytometric analysis of light scattering (data not shown). The ability of the galactosyl transferase treatment of GAV and GAiV to completely inhibit HGAC 39.G3 mAb binding (Fig. 4*b* and *c*) suggests that the bacterial GlcNAc residues available for antibody binding are largely overlapping with those available for enzymatic modification and lectin binding. These results, in conjunction with the ability of free GlcNAc to completely block the binding of the HGAC 39 mAb to the bacteria (3) (data not shown) and the inability of isotype-matched control proteins to bind significantly to the bacteria, suggest that the HGAC 39 mAb are interacting directly with the bacteria solely through V modules.

We compared the relative abilities of affinity-purified HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, and $F(ab')_2$ fragments derived from HGAC 39.G3 to bind to the three bacterial strains using flow cytometry. FITC-conjugated goat antibodies, specific for mouse κ constant domains, were used to detect mAb bound to the bacteria. It is evident that the IgG3 mAb binds significantly better to either GAV or GAiV than the other antibody species tested (Fig. 5), even though all of these preparations exhibited comparable abilities to bind to beads previously coated with anti-mouse κ antibody (Fig. 5*a, inset*). We previously have shown that IgG3 mAb, specific for group A carbohydrate, that bind cooperatively to bacteria do not bind cooperatively to anti-idiotypic or anti-isotypic antibodies (24). The binding of the

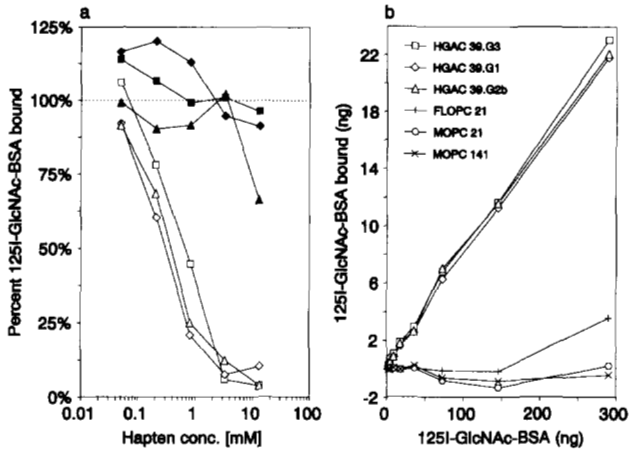

FIGURE 2. Binding of multivalent Ag to immobilized V region-identical mAb. *a,* Percentage inhibition of the binding of 235 ng radiolabeled $GlcNAc_{16}$-BSA to solid-phase mAb HGAC 39.G3 (*squares*), HGAC 39.G1 (*diamonds*) and HGAC 39.G2b (*triangles*) by GlcNAc (*open symbols*) or GalNAc (*closed symbols*). Binding of radiolabeled $GlcNAc_{16}$-BSA in absence of hapten is indicated by *dotted line. b,* Binding of radiolabeled GlcNAc-BSA to solid-phase antibodies. The background binding of radiolabeled GlcNAc-BSA to solid-phase BSA was subtracted from the binding of radiolabeled GlcNAc-BSA to the immobilized mouse antibody. For both panels, an excess of mouse antibodies was bound to adsorbed polyclonal goat antibody specific for the relevant mouse IgG subclass.

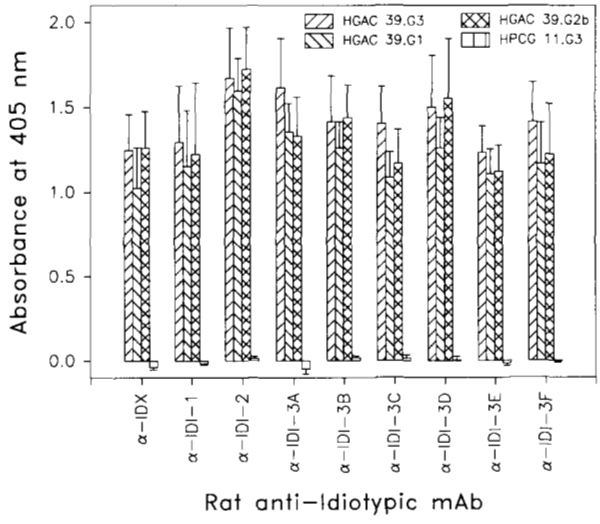

FIGURE 3. Binding of biotin-conjugated HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, and HPCG 11.G3 to nine rat anti-idiotypic mAb by ELISA. HPCG 11.G3 is a control mouse IgG3 κ mAb with specificity for phosphorylcholine. These mAb preparations bound comparably to solid-phase goat antibody specific for mouse κ C domains. The rat anti-idiotypic mAb were bound to adsorbed mouse mAb specific for rat κ domains. Biotinylated mAb were added at 2 μg/ml. The absorbance values are the means of eight replicates, and the 95% confidence intervals are shown.

three HGAC 39 mAb and the IgG3-derived $F(ab')_2$ fragments to the streptococci was specific, as it was completely

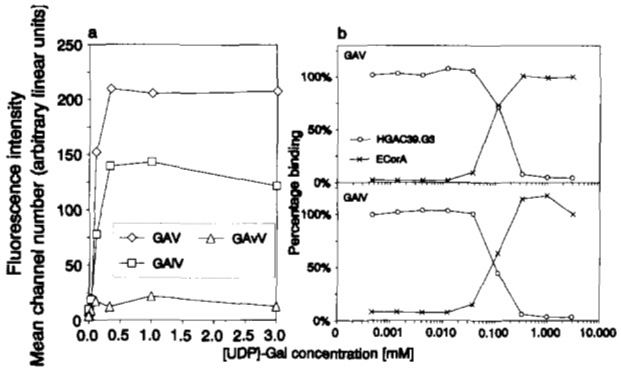

FIGURE 4. Flow cytometric assessment of GlcNAc epitope density on three strains of streptococci. *a,* Binding of biotin-conjugated lectin (100 μg/ml), ECorA, to GAV (*diamonds*), GAiV (*squares*) and GAvV (*triangles*) after treatment of the bacteria with [UDP]-Gal and galactosyl transferase, as a function of [UDP]-Gal concentration. *b,* Percentage binding of ECorA (*crosses,* 100 μg/ml) and biotin-conjugated HGAC 39.G3 (*circles,* 80 μg/ml) to GAV (*top*) and GAiV (*bottom*), pretreated with galactosyl transferase in the presence of a variable amount of substrate, [UDP]-Gal. The binding of the lectin and HGAC 39.G3 to GAvV (data not shown) after enzyme treatment was small compared with the binding to GAV and GAiV. The binding of the biotin conjugates was detected with FITC-avidin (Zymed). The mean values of the fluorescent distributions were converted from the amplified logarithmic scale to a linear scale using a nomogram ($F_{Linear} = 0.48752 \times 1.00782^{F\ Log}$), where F_{Linear} is the mean fluorescent channel number in linear units and F_{Log} is the mean fluorescent channel number in logarithmic units) generated with fluorescent beads of different intensities (Flow Cytometry Standards Corp., Research Triangle Park, NC).

inhibited by free GlcNAc, but not by free rhamnose or GalNAc (3) (data not shown). None of the antibodies bound significantly to GAvV (<15% of the binding to GAV), as assessed by flow cytometry (Fig. 5*b*), although weak (Table I), but reproducible binding was observed by solid-phase ELISA.

The relative difference in binding between intact IgG3 and any of the other antibody species, is consistently greater on GAiV than on GAV. In addition to substantial binding differences between HGAC 39.G3 and the other HGAC 39 antibody species (HGAC 39.G1, HGAC 39.G2b, HGAC 39.G3-derived F(ab')2 fragments), Fig. 5*a* also shows that, on GAV, the HGAC 39.G3-derived F(ab')2 fragments bind substantially better than the intact IgG1 and IgG2b mAb. It is particularly interesting that whereas the IgG3 mAb reproducibly binds more effectively to GAiV than to GAV, HGAC 39.G1, HGAC 39.G2b, and HGAC 39.G3-derived $F(ab')_2$ fragments bind better to GAV than to GAiV. A second IgG1 mAb, independently derived by sequential sublining from HGAC 39.G3, also binds better to GAV than to GAiV (data not shown). Results similar to those obtained with purified HGAC 39 mAb preparations were obtained with serum-free hybridoma supernatants containing, respectively, the IgG3, IgG1, and IgG2b mAb using FITC-

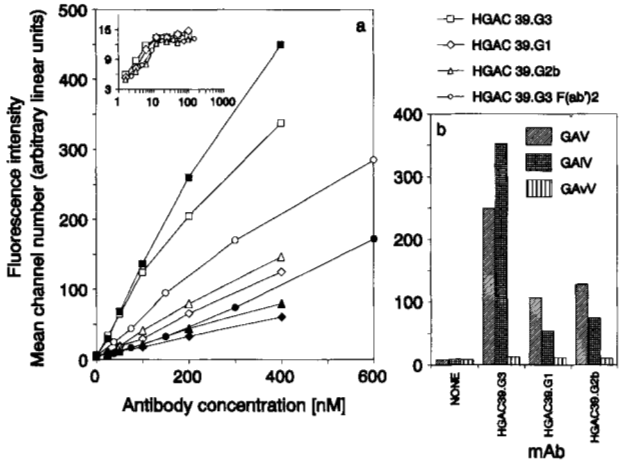

FIGURE 5. IgG subclass-associated differences in binding to GAV and GAiV, by flow cytometry. *a,* Binding at 37°C of HGAC 39.G3 (*squares*), HGAC 39.G1 (*diamonds*), HGAC 39.G2b (*triangles*), and HGAC 39.G3-derived $F(ab')_2$ fragments (*circles*) to GAV (*open symbols*) or GAiV (*closed symbols*). The binding of isotype-matched myeloma proteins (FLOPC 21, MOPC 21, MOPC 141) at 400 nM to GAV and GAiV was less than 11.5 mean fluorescence units. *Inset,* The binding of antibody to beads coated with goat polyclonal antibody specific for mouse κ determinants. The binding of the label in the absence of HGAC 39 antibody was 3.3 mean channel numbers. The binding to beads is a measure of the relative functional antibody concentration used in each experiment. *b,* The binding at 4°C of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b at 15 μg/ml to GAV, GAiV, and GAvV (these data are from a different experiment than those presented in part *a*). The fluorescence intensity is the mean channel number (arbitrary units) of between 17,700 and 19,990 fluorescent events that was converted to a linear scale using the standard curve described in the legend of Figure 4.

conjugated goat anti-mouse κ or anti-mouse IgG for detection (data not shown). Therefore, it is unlikely that the binding advantage of the IgG3 mAb (relative to the IgG1 and IgG2b mAb) is dependent on another molecule (that might co-purify with the mAb by affinity chromatography), such as a subclass-specific rheumatoid factor, or is the result of IgG subclass-biased detection associated with the labeled κ-specific antibody.

Comparisons of relative binding abilities of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b, were also carried out by ELISA with the bacterial preparations adsorbed to the solid phase (Table I). The IgG3 mAb exhibited greater binding than the IgG1 and IgG2b mAb on all three targets, with the greatest subclass-associated differences in binding occurring on GAiV, as was the case using flow cytometry. Similar results were obtained with serum-free hybridoma supernatants containing HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b (data not shown). However, all of the antibody species exhibited an equivalent ability to compete with a biotin-conjugated κ mAb for binding to solid-phase anti-mouse κ antibody (data not shown). The IgG1 and IgG2b mAb, cross-linked by the appropriate goat subclass-specific antibody, bound strongly and specifically to GAV, GAiV, and GAvV (data not shown). Thus, the IgG1 and IgG2b V regions are clearly capable of binding GlcNAc epitopes on all three strains.

Binding of mAb to GlcNAc-BSA conjugates with different epitope densities

We have shown that the three streptococcal strains used for the comparisons of the HGAC 39 mAb differ in average epitope density, but it is conceivable that they differ in other ways that might influence the relative binding of the HGAC 39 mAb. Therefore, we wanted to determine if the IgG3 mAb would bind differently than the V domain-identical IgG1 and IgG2b mAb to a synthetic GlcNAc-bearing Ag. Our initial experiments used a commercially available (Sigma) GlcNAc-BSA conjugate, carrying approximately 16 GlcNAc residues/BSA molecule. In Fig. 6*a,* it is clear that, at 4°C, HGAC 39.G3 binds much more effectively to this solid-phase Ag than either HGAC 39.G1 or HGAC 39.G2b, consistent with the results obtained using the three streptococcal vaccine preparations.

To assess the influence of epitope density on the differential in relative binding between the IgG3 mAb and the IgG1 and IgG2b mAb, we synthesized a set of GlcNAc-BSA conjugates bearing, on average, 50, 20, 10, 5, and 1 GlcNAc residues/BSA molecule. It should be noted that the linkers attaching the GlcNAc residues to the BSA molecules differed for the conjugates we synthesized versus the conjugate obtained commercially, and this difference in linker may be related to the less effective binding by the HGAC 39 mAb to the conjugates we prepared. The results in Fig. 6*b* indicate that although the IgG3 mAb bound more than the IgG1 and IgG2b mAb to the $GlcNAc_{50}$-BSA, the IgG2b mAb was consistently the most effective in binding to the GlcNAc-BSA conjugates with lower GlcNAc substitution ratios. These differences are statistically highly significant (Fig. 6 legend). The IgG1 mAb was consistently the least effective mAb in binding to all of the GlcNAc-BSA conjugates. Thus, using GlcNAc-BSA conjugates, as well as streptococci, the HGAC 39 mAb exhibit different apparent specificities for multivalent Ag of varying epitope density.

Effect of temperature on antibody binding to bacteria and GlcNAc-BSA

We have shown previously that several IgG3 mAb specific for group A carbohydrate, including HGAC 39.G3, bind significantly better at 4°C than at 24°C or 37°C to a synthetic, solid-phase Ag, $GlcNAc_{16}$-BSA (25). This result raises the issue of the physiologic relevance of IgG subclass-related binding differences. Therefore, we investigated whether temperature significantly affects the binding of the HGAC 39 mAb, of the various subclasses, to the three streptococcal strains. The results of ELISA at 4°C, and

Table I
Binding[a] of HGAC 39 mAb and myeloma proteins to three streptococcal strains by ELISA. The 95% confidence intervals of the OD 405 nm (absorbance units) are shown

Antibody	Conc. [μg/ml]	GAV		GAiV		GAvV	
		Mean	95%	Mean	95%	Mean	95%
HGAC 39.G3	10.0	1.429	±0.248	2.488	±0.159	–[b]	–
	100.0	–	–	–	–	0.819	±0.154
HGAC 39.G1	10.0	0.812	±0.100	0.733	±0.078	–	–
	100.0	–	–	–	–	0.035	±0.008
HGAC 39.G2b	10.0	1.100	±0.095	0.847	±0.060	–	–
	100.0	–	–	–	–	0.064	±0.015
FLOPC 21	10.0	0.015	±0.023	0.017	±0.017	–	–
	100.0	–	–	–	–	0.024	±0.015
MOPC 21	10.0	0.013	±0.009	0.016	±0.015	–	–
	100.0	–	–	–	–	0.043	±0.022
MOPC 141	10.0	0.024	±0.012	0.056	±0.029	–	–
	100.0	–	–	–	–	0.060	±0.022

[a] Mouse antibody was incubated with bacteria adsorbed to the solid phase. Antibody binding was measured following addition of alkaline phosphatase-conjugated goat antibody specific for mouse κ-constant domains. Background binding of the label to the solid-phase bacterial strains, in the absence of mouse antibody, has been subtracted.
[b] Dash indicates not determined.

37°C, using GAV, GAiV, and GAvV (Fig. 7) indicate no significant temperature-dependent differences in binding for any of the antibodies, except that the binding of HGAC 39.G3 to GAvV does not reach a plateau at 37°C within the assay concentration limits. By flow cytometry (data not shown), the binding of each of the mAb to GAV or GAiV did not vary significantly with temperature when tested at 4°C and 37°C. There is no detectable binding to GAvV for any of the mAb by flow cytometry at any of the three temperatures.

Given our earlier demonstration (25) that HGAC 39.G3 (and a few other IgG3 mAb) bound to solid-phase GlcNAc-BSA in a temperature-dependent manner, it was of interest to determine if binding by HGAC 39.G1 and HGAC 39.G2b would exhibit such a dependence on temperature. In Figure 8, we show that HGAC 39.G3 binds substantially better to $GlcNAc_{16}$-BSA at 4°C than at 24°C or 37°C. However, HGAC 39.G1 and HGAC 39.G2b bind equivalently, at all three temperatures tested, to either commercially obtained $GlcNAc_{16}$-BSA or our $GlcNAc_{10}$-BSA conjugate. It is interesting that the binding of HGAC 39.G3 to $GlcNAc_{10}$-BSA is not significantly influenced by temperature. Therefore, with respect to the various GlcNAc-BSA conjugates we have used, there is a correlation between HGAC 39.G3 binding better than HGAC 39.G1 or HGAC 39.G2b and HGAC 39.G3 binding better at lower temperatures than at higher temperatures. Both of these phenomena occur when the solid-phase target is the commercial conjugate, $GlcNAc_{16}$-BSA, or our $GlcNAc_{50}$-BSA (data not shown for the effect of temperature). When the target is either $GlcNAc_{10}$-BSA or $GlcNAc_5$-BSA, HGAC 39.G3 binds equivalently at different temperatures [$GlcNAc_{10}$-BSA (Fig. 8); $GlcNAc_5$-BSA (data not shown)], and HGAC 39.G3 does not bind better than HGAC 39.G2b, although, HGAC 39.G3 does bind better than HGAC 39.G1 (Fig. 6).

Discussion

These results confirm and extend our previous observations on the contributions of C_H domains to IgG subclass-associated differences in the binding of antibodies to multivalent Ag, such as bacterial surfaces (3, 26). The major conclusion from the current study is that the apparent specificities, as well as the apparent functional affinities, of IgG antibodies, can be affected by structural differences in C_H domains. This conclusion depends critically on our demonstration that the V regions of the IgG3, IgG1, and IgG2b mAb, specific for GlcNAc residues of GAC, are indistinguishable.

The assertion that C_H domains can influence apparent antibody specificity is based on the observation that differences in average epitope density associated with streptococci (Fig. 4) or with GlcNAc-BSA conjugates are correlated with variations in the relative binding of HGAC 39.G3 versus HGAC 39.G1 or HGAC 39.G2b (Figs. 5–8, Table I). Similar differences in relative binding are seen between intact HGAC 39.G3 and HGAC 39.G3-derived $F(ab')_2$ fragments (3, 25), suggesting that structural differences in Fc regions (C_H2 and C_H3 domains) in particular, can influence the apparent specificities of antibodies. Therefore, these results suggest that epitope spacing, as well as epitope fine structure, can contribute to the ability of antibodies to discriminate among multivalent Ag.

Recent studies with DNA-binding proteins provide additional evidence that target site spacing can serve as an important determinant of specificity in the context of recognition by proteins capable of intermolecular cooperativity (27–29). Furthermore, in analogy to our conclusion that alterations in Fc region structure can alter apparent antibody specificity, mutations in a region of a DNA-binding protein involved primarily in protein-protein

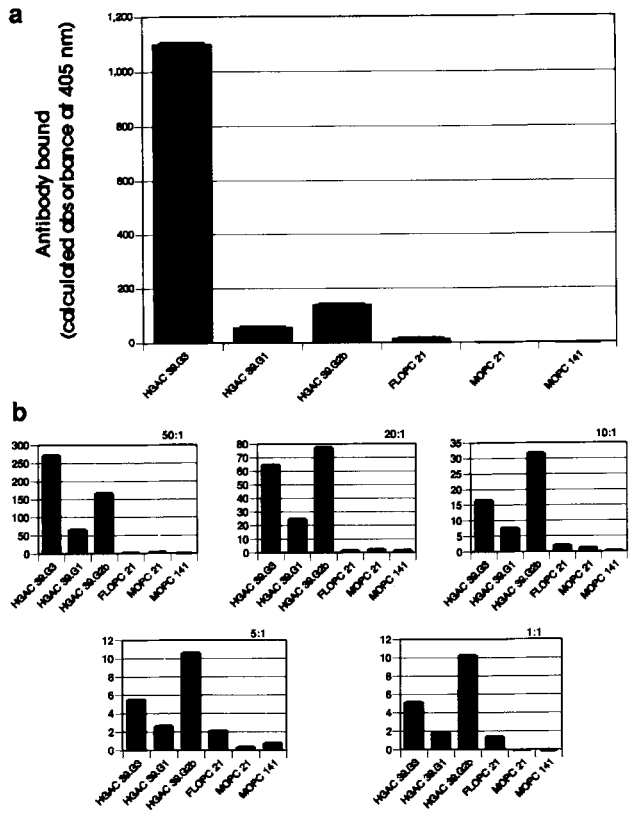

FIGURE 6. Binding, at 4°C, of V region-identical mAb to commercially obtained $GlcNAc_{16}$-BSA (*a*) or to GlcNAc-BSA conjugates with different substitution ratios of GlcNAc to BSA (*b*), by solid-phase ELISA. The structures of the linkers for the commercially obtained GlcNAc-BSA and the GlcNAc-BSA conjugates we synthesized are different. Each GlcNAc-BSA conjugate was adsorbed to the wells at 5 μg/ml. Bound antibody was detected with alkaline phosphatase-labeled polyclonal antibody specific for mouse κ constant domains (FisherBiotech). Because of the wide range of binding activities, depending on the antibody and the Ag, antibody binding is presented as the calculated binding at a theoretical time of 10,000 min after addition of substrate. This approach permits all of the mAb-Ag conjugate combinations to be compared in a single assay. The absorbance values were repeatedly recorded for the wells (nine replicates for the HGAC 39 mAb and three replicates for the myeloma proteins), and they were found to be linear for at least 5 h. A linear regression equation (method of least squares) was generated for each mAb-Ag combination and was used to calculate the expected absorbance at 10,000 min. The coefficient of correlation was at least 0.99 for each of the 36 equations. The 99% confidence intervals are shown as black bars on top of each histogram.

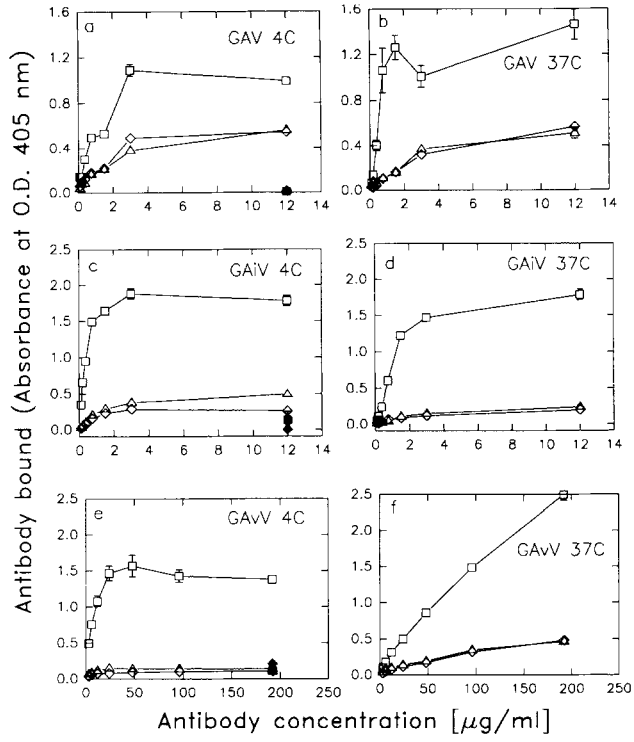

FIGURE 7. Effect of temperature on IgG subclass-associated differences in binding to GAV, GAiV, and GAvV by ELISA. The binding of HGAC 39.G3 (*open squares*), HGAC 39.G1 (*open diamonds*), HGAC 39.G2b (*open triangles*), and isotype-matched myeloma proteins, FLOPC 21 (*closed squares*), MOPC 21 (*closed diamonds*) and MOPC 141 (*closed triangles*), to GAV (*a, b*), GAiV (*c, d*), GAvV (*e, f*) at 4°C (*a, c, e*) and 37°C (*b, d, f*). The SEM is provided for each data point; however, in most cases, the confidence intervals are narrower than the data point symbols. For each bacterial strain, the binding data were recorded after the same substrate development time. The substrate absorbance readings were determined after an incubation of 70 min for GAV, 35 min for GAiV and 69 min for GAvV.

interactions can alter the specificity of the molecule for DNA elements involved in genetic regulation (27).

Accounting for the better binding of HGAC 39.G3 to GAiV versus GAV, given that HGAC 39.G1 and the other antibody species tested (as well as ECorA, after galactosylation) all tend to bind better to GAV than to GAiV, is challenging given our present state of knowledge of the systems under study. Although our lack of detailed knowledge about the respective epitope distributions on GAV and GAiV hinders the construction of a definitive explanation for the observed patterns of binding, we can speculate that the variations in mAb binding relate to differences in the probability of monogamous bivalent binding, the probability of inter-antibody association, and the number of sites effectively utilized by the different antibody species. Experiments utilizing surface plasmon resonance (30) to measure the binding of HGAC 39 mAb to GlcNAc-BSA (L. J. N Cooper, D. Robertson, R. Granzow, and N. Greenspan in preparation) suggest that the number of epitopes is effectively greater for the IgG3 mAb than for the IgG1 and IgG2b mAb or the IgG3-derived $F(ab')_2$ fragments, although we cannot directly extrapolate these findings to the interactions between HGAC 39 mAb and GAV or GAiV. Such a disparity in effective epitope number could result from the conjunction of two factors: 1) the HGAC 39 mAb exhibit very low intrinsic affinities (50% inhibition by GlcNAc in the mM range), such that IgG molecules binding

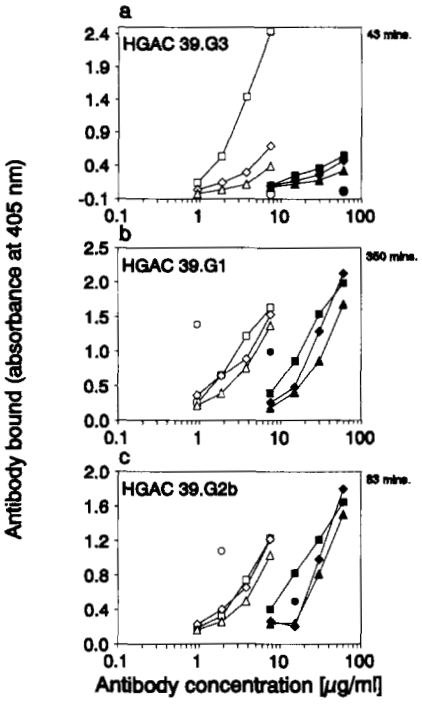

FIGURE 8. Effect of temperature on the binding of affinity-purified V region-identical IgG3, IgG1, and IgG2b mAb to different GlcNAc-BSA conjugates by ELISA. Binding of HGAC 39.G3 (*a*), HGAC 39.G1 (*b*), and HGAC 39.G2b (*c*), to wells coated with 5 μg/ml of $GlcNAc_{16}$-BSA (*open symbols*; Sigma) or $GlcNAc_{10}$-BSA (*closed symbols*) at 4 (*squares and circles*), 24 (*diamonds*), and 37°C (*triangles*). The *circular symbols* represent the absorbance values for FLOPC 21 (*a*) or HGAC 39.G3 (*b* and *c*). The time points (in minutes), after addition of substrate, at which the absorbance values were measured are indicated. The time points are different because the experiment was optimized to compare the binding by each antibody at different temperatures, not to compare the binding of one mAb with another mAb. Background binding of alkaline phosphatase-conjugated goat anti-mouse κ antibody to the solid phase was subtracted from the graphed absorbance values.

independently through a single paratope would dissociate rapidly, and 2) IgG subclass-related Fc region-dependent interactions. Thus, IgG3 molecules, but not IgG1 and IgG2b mAb and IgG3-derived $F(ab')_2$ fragments, might be able to bind to regions of the Ag surface where only one Fab arm (of a single IgG molecule) can make effective contact to an epitope, because only the IgG3 molecules bind cooperatively. This argument is consistent with the very weak binding to GAV of Fab fragments derived from HGAC 39.G3 (24).

We do not believe that the basis for the superior binding exhibited by HGAC 39.G3 in comparison with HGAC 39.G1 or HGAC 39.G2b resides in direct interactions between nonparatopic sites on HGAC 39.G3 and the cell walls of the bacteria. First, as shown previously, free GlcNAc can completely inhibit binding of HGAC 39 mAb to GAV (24) and GAiV (3). Second, covalent modification of GlcNAc residues by galactosyl transferase (addition of galactose) eliminates binding by HGAC 39.G3 (Fig. 4*b* and *c*). This enzymatic reaction is supposed to be completely specific for terminal nonreducing GlcNAc residues. Third, isotype-matched mAb of irrelevant specificity do not bind significantly to GAV, GAiV, or GAvV (3) (Fig. 5 legend). Fourth, the HGAC 39 mAb bind specifically to GlcNAc-BSA (Figs. 2, 6, and 8), and IgG subclass-associated binding differences, similar in some respects to those seen with the streptococci, are observed with solid-phase GlcNAc-BSA (Figs. 6 and 8). Furthermore, we do not believe that substantial amounts of HGAC 39.G3 can remain bound to the streptococci indirectly through Fc-Fc interactions with previously bound HGAC 39.G3, and independent of epitope-paratope interaction for the newly bound IgG3 molecule, as physical interaction between IgG3 mAb of distinct specificity requires the presence of both cognate epitopes on the target surface (31). However, at present, we cannot absolutely rule out the existence of a second (non-paratopic) interaction between HGAC 39.G3 and the bacterial surface that only contributes to mAb binding in the presence of paratope-epitope interactions. A bacterial structure mediating such an interaction would have to be: 1) resistant to heating at 56°C for 3 h followed by pepsin treatment for 2 h, 2) expressed at a higher level on GAiV than on GAV, and 3) and have a site specificity that does not interfere with IgG3 Fc-Fc interactions.

A feature of the IgG C_H domains that might influence the specificity of binding, aside from an ability to self-associate is segmental flexibility, which has been conjectured to be a determinant of IgG functional affinity (32). Currently, however, there are no definitive data correlating antibody segmental flexibility with strength of binding to multivalent Ag. The available data on segmental flexibility of mouse IgG subclasses suggests that IgG3 and IgG1 are relatively inflexible, IgG1 being slightly less flexible than IgG3, whereas, IgG2b is the most flexible (33). Because the IgG1 and IgG2b subclasses span the range of mouse IgG segmental flexibility, and given that HGAC 39.G1 and HGAC 39.G2b display no major differences in relative binding to GAV versus GAiV, it is unlikely that segmental flexibility plays a primary role in determining differences in apparent specificity of the HGAC 39 mAb for the different strains of streptococci.

However, we do have some evidence that segmental flexibility, or unidentified factors that vary with IgG subclass (34), can affect antibody binding to and apparent specificity for the streptococci. IgG3-derived $F(ab')_2$ fragments, which do not display detectable cooperativity (3), exhibit relatively greater binding to GAV (by flow cytometry) than intact HGAC 39.G1 and HGAC 39.G2b mAb (Fig. 5*a*), and HGAC 39.G3 binds better to GAvV (by ELISA; Table I) than HGAC 39.G1 and HGAC 39.G2b, despite a lack of detectable IgG3 cooperativity on this target (data not

shown). One possible explanation for the difference between the IgG3-derived $F(ab')_2$ fragments and the IgG1 and IgG2b mAb, is that the IgG1 and IgG2b mAb may exhibit a degree of Fc region-dependent negative cooperativity. Nygren and Stenberg (34) have speculated on the occurrence of negative as well as positive cooperativity in the binding of antibodies to surfaces. Currently, we have no definitive evidence to support this possibility, nor can we definitively explain why such negative cooperativity would be apparent on GAV but not GAiV.

The results of comparisons of the HGAC 39 mAb binding to GlcNAc-BSA conjugates can be explained largely on the basis of IgG subclass-related variation in cooperative binding and segmental flexibility. At high epitope density, the IgG3 mAb may have an advantage because of intermolecular cooperativity, whereas at lower epitope densities, intermolecular interactions may become less likely and the importance of monogamous bivalent binding by each individual IgG molecule may increase, given that these mAb have low intrinsic affinities for GlcNAc epitopes. As IgG2b is believed to be the most flexible of the mouse IgG subclasses (33), it is reasonable to suppose that under conditions of relatively low epitope density the IgG2b mAb binds more effectively than the relatively rigid IgG3 and IgG1 mAb, by virtue of a greater probability of monogamous bivalent interaction. The ability of HGAC 39.G2b to bind more effectively than HGAC 39.G3 to some GlcNAc-BSA conjugates indicates that the IgG subclass-associated differences in binding to the bacteria (IgG3 > IgG2b, IgG1) are not likely to be explained by a bias, related to IgG subclass, in the ability of labeled anti-mouse κ antibodies to detect bound HGAC 39 mAb.

The lack of a significant effect of temperature on the binding of any of the three HGAC 39 mAb to GAV, GAiV, or GAvV (Fig. 7, and data not shown) is in striking contrast to the effect of temperature on the binding of HGAC 39.G3 to the synthetic glycoconjugate, $GlcNAc_{16}$-BSA (24) (Fig. 8). HGAC 39.G3 clearly bound progressively better to solid-phase $GlcNAc_{16}$-BSA at 37°C, 24°C, and 4°C (Fig. 8). Therefore, it is important to note that the better binding of HGAC 39.G3 (compared with HGAC 39.G1 and HGAC 39.G2b) to bacteria was present at physiologic temperatures (Fig. 7). In this context, it is also of interest that the magnitude of the cooperative effect is generally greater on binding to bacteria (GAV and GAiV) than on binding to GlcNAc-BSA (N. S. Greenspan and L. J. N. Cooper, unpublished observations). Perhaps a more optimal epitope distribution on the bacteria fosters greater Fc-Fc interaction, thereby minimizing the effect of lower temperature in facilitating Fc region-dependent contacts. Another factor that might contribute to the variable effect of temperature on binding of the IgG3 mAb to different Ag is Ag-associated differences in solvation.

In conclusion, when antibody-Ag interaction involves antibodies capable of intermolecular cooperative binding, structural determinants not normally involved in determining specificity can significantly influence the relative binding to targets of varying epitope density. This conclusion suggests that it may be possible to engineer antibodies, through alterations in the C_H domains, so that they exhibit optimal binding to targets expressing particular distributions of the cognate epitope (26).

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