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H CHAIN C DOMAINS INFLUENCE THE STRENGTH OF BINDING OF IgG FOR STREPTOCOCCAL GROUP A CARBOHYDRATE[1]

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We have produced a panel of murine anti-streptococcal mAbs, expressing identical V domains and different H chain C domains, corresponding to the IgG3, IgG1, and IgG2b subclasses. We have used these mAb to evaluate the role of IgG subclass-specific C region determinants in modulating the interaction between antibody and the bacterial surface. We report, for the first time, that V region-identical murine IgG of different subclasses exhibit substantial differences in binding to specific Ag; IgG3 mAb binds more strongly to streptococci than the IgG1 and IgG2b mAb or IgG3-derived F(ab')$_2$ fragments. Furthermore, the IgG3 mAb binds cooperatively to the bacteria, whereas the IgG1, IgG2b, and IgG3-derived F(ab')$_2$ fragments do not exhibit significant cooperativity, which suggests that differences in Fc region structure can affect antibody binding to multivalent Ag by modulating the potential for cooperative binding. These results suggest a plausible mechanism by which murine IgG3 could be more effective, than other antibodies bearing identical V domains, but of different γ-subclass, in mediating bacterial immunity.

The strength of IgG antibody binding is thought to depend on intrinsic affinity, which is determined by the V domains, and on segmental flexibility, which is controlled by the hinge and C_H1 regions (1–3). However, there is no definitive evidence relating differences in segmental flexibility to differences in strength of binding.

We have generated a set of IgG mAb,[3] expressing identical variable domains but different H chain C domains, to test the influence of IgG H chain C domains on the strength of mAb binding. IgG subclass-associated binding differences have been reported for mouse, rat, and human serum antibodies (4–6), but the structural causes for these differences are unclear. Previous analyses of spontaneous IgG subclass switch variants (7), or V domain-identical chimeric IgG antibodies (8), have not revealed IgG subclass-associated differences in intrinsic affinity. These results have led some investigators to assume that IgG antibodies expressing identical V domains, and therefore, identical intrinsic affinities, will necessarily exhibit identical binding, even to multivalent Ag (9). We now describe an exception to this generalization.

IgG3 mAb specific for the GlcNAc[4] residues of GAC have previously been shown to bind to heat-killed, pepsin-digested group A streptococci (GAV) and to GlcNAc-BSA by an Fc region-dependent cooperative mechanism (10–12). We hypothesize that this cooperative mechanism contributes to the strength of binding of the IgG3 mAb for GAC, and that cooperative binding is modulated by differences in Fc region structure, such as are found among the murine IgG subclasses. Thus, subclass-associated differences in the Fc region might result in differences in strength of binding of antibody for Ag. These considerations, in conjunction with the fact that IgG3 is the IgG subclass predominantly elicited by immunization with GAV (13, 14), prompted us to determine whether IgG mAb expressing identical V domains but different H chain C domains bind equivalently to group A streptococci.

MATERIALS AND METHODS

Antibodies. HGAC 39.G3 (IgG3 κ previously referred to as HGAC 39) is a murine mAb that recognizes the GlcNAc residues of the streptococcal cell wall polysaccharide, GAC (15). IgG1 (HGAC 39.G1) and IgG2b (HGAC 39.G2b) variants of HGAC 39.G3, were produced by selecting spontaneous isotype switch variants with isotype-specific antisera (FisherBiotech, Pittsburgh, PA), by ELISA (16). GAC-specific mAb were affinity-purified on GlcNAc-conjugated agarose (Sigma Chemical Co., St. Louis, MO) from hyridoma culture supernatant. Isotype-matched myeloma proteins with irrelevant specificity (Sigma; FLOPC 21, IgG3; MOPC 21, IgG1; MOPC 141, IgG2b) were used as nonspecific Ig controls.

Streptococcal bacteria. The Ag used in the present study was GAiV which consists of heat-killed pepsin-digested group A intermediate streptococci. Group A intermediate streptococci were derived from group A streptococci by mouse passage and possess a cell wall polysaccharide (group A intermediate carbohydrate) with a lower ratio of GlcNAc to rhamnose than is the case for the parental strain carbohydrate (17). The strain of group A intermediate streptococci used was originally obtained from M. McCarty, Rockefeller University, New York, NY.

Flow cytometry to measure antibody binding. GAiV (~0.6 OD_{650} U/tube) or beads (~1.45×10^8 particles) were incubated overnight at 37°C with equivalent concentrations (OD_{280}) of HGAC 39.G3, HGAC 39.G1 and HGAC 39.G2b or isotype-matched myeloma proteins. The beads (Polysciences, Warrington, PA; 1 μm carboxylate polystyrene microparticles) were previously adsorbed with polyclonal goat antibody to mouse κ determinants (Fisher Biotech) and blocked with 1% BSA/PBS. GAiV and beads were washed twice with 1% BSA/PBS and stained overnight at 4°C with FITC-conjugated goat antibody to

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[3] HGAC 39.G3 was generated through the Mallinkrodt-Washington University Hybridoma Contract.

[4] Abbreviations used in this paper: GlcNAc, *N*-acetyl-D-glucosamine; GAC, streptococcal group A carbohydrate; GAV, group A vaccine; GAiV, group A intermediate vaccine.

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mouse κ determinants (50 μg/ml; FisherBiotech). After two further washes with 1% BSA/PBS, the GAiV and bead preparations were fixed in PBS containing 0.4% formaldehyde.

The preparations were analyzed with an Ortho Cytofluorograph IIs (Becton Dickinson, Westwood, MA) by using the 488-nm line of an argon laser at 250 mW. Fluorescence was collected at a 540/20-nm bandpass, and the subsequent signal logarithmically amplified. Approximately 20,000 events were acquired for each sample by using forward and orthagonal light scatter as gating criteria to identify GAiV or beads. The mean values of the distributions for the various Ig species were converted from a logarithmic scale to a linear amplified scale by using a nomogram generated with fluorescent beads of different intensities (Flow Cytometry Standards Corp., Research Triangle Park, NC). Some preparations of GAiV and beads were remeasured at the end of an experiment to assess any fluctuations in laser power or flow rates, and no significant alterations were found.

ELISA to measure antibody binding. GAiV (0.016 OD_{650} U/well) was dried on 96-well polyvinyl chloride microtiter wells (Dynatech, Pittsburgh, PA). Equivalent concentrations (OD_{280}) of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b or isotype-matched myeloma proteins were incubated with Ag overnight at 4°C. Binding was detected with alkaline phosphatase-conjugated polyclonal goat anti-mouse κ, and nonspecific binding of the labeled antibody to the solid-phase bacteria was subtracted from the mean mAb binding measurements. Equivalent concentrations of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b comparably inhibited binding of HGAC 39.G1-biotin to polyclonal goat anti-mouse κ (data not shown).

Hapten inhibition. In the ELISA, 5 μg/ml of HGAC 39.G3, HGAC 39.G1 and HGAC 39.G2b mAb were incubated in the presence or absence of GlcNAc overnight at 4°C. The binding of the mAb to solid-phase GAiV, 12 replicates per hapten concentration, were detected with alkaline phosphatase-conjugated polyclonal goat anti-mouse κ. In the flow cytometric experiment, HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, HGAC 39.G3-derived $F(ab')_2$ fragments, and HGAC 39.G1-derived $F(ab')_2$ fragments, at 60 μg/ml, were each incubated with GAiV at 37°C in the presence of GlcNAc or rhamnose. The mean value of the linear channel number (arbitrary units) was measured as described above (flow cytometry methods). In both experiments, the 50% inhibition point was determined relative to the binding of the anti-GAC mAb in the absence of hapten.

RIA. The protocol was as described for binding of [^{125}I]HGAC 39.G3 to solid-phase GAV or GlcNAc-BSA (10, 11). Briefly, GAiV was adsorbed to round-bottomed 96-well plates, and the wells were blocked. Approximately 30 ng of [^{125}I]HGAC 39.G3, [^{125}I]HGAC 39.G1, or [^{125}I] HGAC 39.G2b, labeled by chloramine T method (18), were added per well in the presence of an unlabeled antibody and incubated overnight at room temperature. The plates were washed, and the radiolabel bound to each well was counted in an automated gamma counter (Pharmacia-LKB, Piscataway, NJ). The unlabeled antibody was assessed for the ability to inhibit or enhance the binding of labeled mAb.

RESULTS

Binding differences by flow cytometry and ELISA. HGAC 39.G3 (IgG3 κ) is a murine mAb specific for GlcNAc residues of the group A streptococcal cell wall polysaccharide. Hybridomas secreting HGAC 39.G1 and HGAC 39.G2b were derived by sib selection (16) from the hybridoma secreting HGAC 39.G3. Although we have been unable to formally measure the intrinsic affinities (estimated at 10^3 to 10^4 L/M; see Fig. 3) (19) of the three HGAC 39 mAb, because of limitations of such procedures with antibodies of such low affinities, experiments we will describe in detail separately (L. J. N. Cooper et al., manuscript in preparation) demonstrate that: 1) the nucleotide sequences encoding, respectively, the V_H and V_L domains of HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b are identical, and 2) the reactivities of the three mAb with soluble radiolabeled GlcNAc-BSA and anti-idiotypic mAb are comparable. Therefore, we believe it is likely that the three HGAC 39 mAb express identical intrinsic affinities.

In Figure 1, *a* and *b*, binding of IgG3, IgG1, and IgG2b anti-GAC mAb and HGAC 39.G3 $F(ab')_2$ fragments to

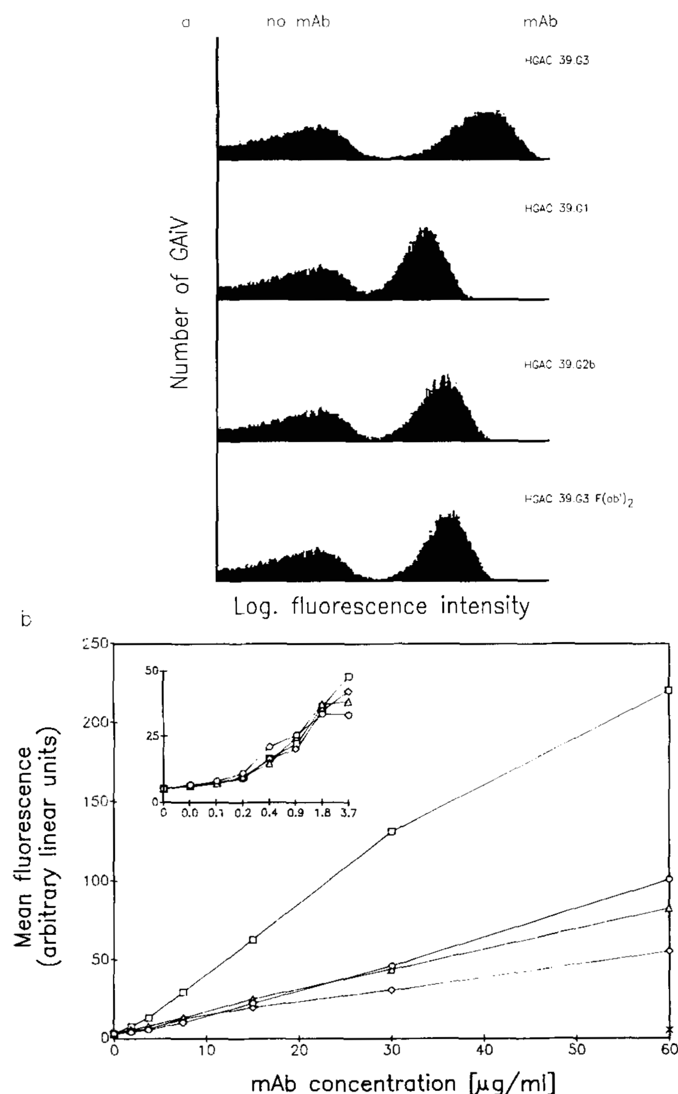

Figure 1. Binding of HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, and HGAC 39.G3-derived $F(ab')_2$ fragments by indirect flow cytometry. *a*, histograms (logarithmic arbitrary units) for HGAC 39.G3, HGAC 39.G1, HGAC 39.G2b, and HGAC 39.G3-derived $F(ab')_2$ binding to GAiV at 60 μg/ml at 37°C. *b*, concentration dependence of mean binding of approximately 20,000 fluorescent events (linear arbitrary units) to GAiV or anti-κ-coated beads (*inset*) for HGAC 39.G3 (□), HGAC 39.G3-derived $F(ab')_2$ fragments (O), HGAC 39.G1 (◇), HGAC 39.G2b (Δ), and mean of three isotype-matched control myeloma proteins (**×**) at 37°C. The 99% confidence intervals were smaller than the symbols used to mark the data points.

GAiV is compared by flow cytometric indirect immunofluorescence, using FITC-labeled polyclonal goat anti-mouse κ antibodies for quantitation. It is apparent that more IgG3 than IgG1, IgG2b, or IgG3-derived $F(ab')_2$ fragments binds to the bacteria in suspension. Independent measurement of the binding of these mAb to goat anti-mouse κ antibody (Fig. 1*b*, *inset*) indicates that the differences in binding to bacteria are not likely to be accounted for by any preference of the labeled second antibody for the IgG3 subclass. HGAC 39.G3 also binds better than HGAC 39.G1 or HGAC 39.G2b as measured by solid-phase indirect ELISA, using alkaline phosphatase-conjugated goat anti-κ antibodies (Fig. 2), or biotin-coupled monoclonal rat anti-mouse κ antibody and streptavidin-alkaline phosphatase (data not shown). A second IgG1 switch variant mAb (independently derived from HGAC 39.G3) gave results similar to those for HGAC

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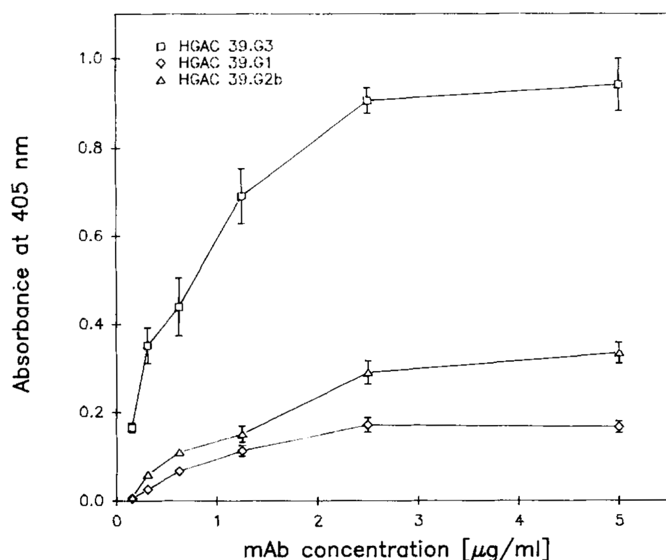

Figure 2. Binding of HGAC 39.G3 (□), HGAC 39.G1 (◇), and HGAC 39.G2b (△) to solid-phase GAiV as measured by ELISA. Plotted points are the means ± SEM of four replicates. Values (OD_{405}) of isotype-matched control myeloma proteins binding to GAiV at 5 μg/ml: FLOPC 21, −0.021 ± 0.004; MOPC 21, −0.021 ± 0.001; MOPC 141, 0.002 ± 0.009.

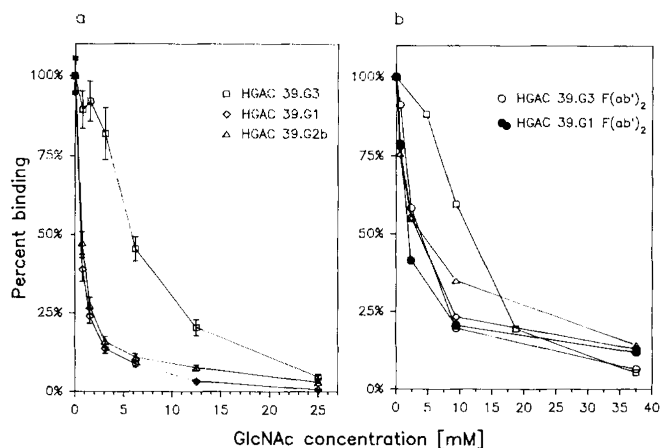

Figure 3. Mean binding (as a percentage of binding of antibodies in the absence of hapten) of HGAC 39.G3 (□), HGAC 39.G3-derived $F(ab')_2$ fragment (○), HGAC 39.G1 (◇), HGAC 39.G1-derived $F(ab')_2$ fragment (●), and HGAC 39.G2b (△), in the presence of GlcNAc, measured by ELISA (mean binding ± SEM of 12 replicates) (*a*), and flow cytometry (mean binding of approximately 20,000 fluorescent events, linear scale) (*b*). Data (including higher GlcNAc concentration flow cytometer values not shown) were fitted to regression equations (correlation coefficients were greater than 0.96) and the hapten concentrations resulting in 50% inhibition of antibody binding were calculated. From this analysis, the concentrations of GlcNAc yielding 50% inhibition are by ELISA (mM): HGAC 39.G3, 5.91; HGAC 39.G1, 0.83; HGAC 39.G2b, 0.73; and by flow cytometry: HGAC 39.G3, 6.99; HGAC 39.G3-$F(ab')_2$, 1.83; HGAC 39.G1, 1.75; HGAC 39.G1-$F(ab')_2$, 1.23; HGAC 39.G2b, 2.30. The differences in the hapten concentrations yielding 50% inhibition of binding for HGAC 39.G3 and the other Ig species were significant ($p < 0.01$) by both ELISA and flow cytometry.

39.G1. In the ELISA and flow cytometry experiments, we also noted small but reproducible differences in binding between HGAC 39.G2b and HGAC 39.G1. The difference in binding between HGAC 39.G2b and HGAC 39.G1 was less than the difference observed between HGAC 39.G3 and either of the other two mAb. Serum-free culture supernatants containing known quantities of the three mAb were also compared, and more HGAC 39.G3 than HGAC 39.G1 or HGAC 39.G2b bound to the bacteria (data not shown). As these supernatants do not contain significant quantities of serum-derived proteins, it is unlikely that the better binding of HGAC 39.G3 is due to the presence of additional molecular species, such as IgG3-specific rheumatoid factors.

We have observed that HGAC 39.G3 binds better than HGAC 39.G1 and HGAC 39.G2b, over a wide range of concentrations, to additional target Ag including: 1) two other strains of group A streptococci (differing in the relative content of GlcNAc and rhamnose in the cell wall polysaccharide), 2) GlcNAc-BSA, and 3) intracellular rat and human proteins bearing O-linked GlcNAc residues (L. J. N. Cooper, et al., manuscript in preparation).

Hapten inhibition. Hapten inhibition studies (Fig. 3) were used to compare the apparent affinities of these mAb and $F(ab')_2$ fragments for the bacteria. The concentrations of GlcNAc inhibiting 50% of the binding of the antibody to bacteria were similar for HGAC 39.G1, HGAC 39.G2b, HGAC 39.G3-derived $F(ab')_2$ fragments, and HGAC 39.G1-derived $F(ab')_2$ fragments. However, by ELISA and by flow cytometry, HGAC 39.G3 required significantly greater concentrations of GlcNAc to inhibit binding by 50%. There was no inhibition of antibody binding by rhamnose (which, along with GlcNAc, is a major constituent of GAiV cell wall polysaccharide) by ELISA or flow cytometry (data not shown). Therefore, HGAC 39.G3 appears to bind more strongly to the streptococci than the other mAb through a mechanism dependent on the $\gamma 3$ Fc region. Furthermore, these results suggest that the binding advantage exhibited by HGAC 39.G3, in comparison with HGAC 39.G1 or HGAC 39.G2b, is not based on greater intrinsic affinity.

Cooperative binding. Since previous studies with GAC-specific IgG3 mAb have revealed one potential mechanism for subclass-associated differences in binding (10, 11), we studied the binding, by RIA, to solid-phase bacteria of radiolabeled HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b mAb in the presence of unlabeled mAb. Unlabeled HGAC 39.G3 substantially enhanced the binding of radiolabeled HGAC 39.G3, but unlabeled HGAC 39.G1 and HGAC 39.G2b exhibited no such enhancement (Fig. 4). The binding of radiolabeled HGAC 39.G3 was inhibited at higher concentrations of unlabeled HGAC 39.G3 (51% inhibition at 56 μg/ml of unlabeled mAb; data not shown) than those shown in Fig. 4*a*. Binding of radiolabeled HGAC 39.G1 or HGAC 39.G2b mAb was not enhanced by unlabeled HGAC 39.G3, HGAC 39.G1, or HGAC 39.G2b (Fig. 4, *b* and *c*). IgG3 and IgG1 antibodies of irrelevant specificity, HGAC 39.G3-derived $F(ab')_2$ fragments, and HGAC 39.G1-derived $F(ab')_2$ fragments also failed to enhance the binding of radiolabeled HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b (data not shown). In addition, the binding of radiolabeled HGAC 39.G3-derived $F(ab')_2$ fragments to solid-phase GlcNAc-BSA was not enhanced by unlabeled HGAC 39.G3 (data not shown). These results are consistent with previous evidence that IgG3, but not IgG2b or IgG1 phosphorylcholine-specific mAb could enhance binding of HGAC 39.G3 to a solid phase expressing both GlcNAc and phosphorylcholine epitopes (12).

If this cooperative binding mechanism (10–12) is used by IgG3, but not by IgG1 or IgG2b mAb, one would predict that IgG3 mAbs would dissociate less rapidly from streptococci than the IgG1 or IgG2b mAbs. Preliminary results demonstrate that HGAC 39.G3 dissociates more slowly from the bacteria than HGAC 39.G1 or HGAC 39.G2b

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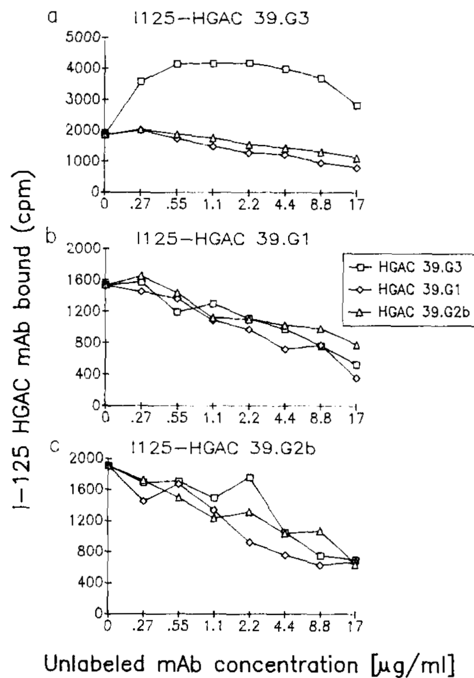

Figure 4. The effect of unlabeled HGAC 39.G3 (□), HGAC 39.G1 (◇), and HGAC 39.G2b (Δ) on the binding (cpm) of [^{125}I]-labeled HGAC 39.G3 (*a*), [^{125}I]-labeled HGAC 39.G1 (*b*), and [^{125}I]-labeled HGAC 39.G2b (*c*) to solid-phase GAiV.

(data not shown). HGAC 39.G3 also dissociated less rapidly than HGAC 39.G1 and HGAC 39.G2b on a different streptococcal group A strain (data not shown).

DISCUSSION

We have demonstrated, for the first time, the influence of H chain C regions, especially the CH_2 and CH_3 domains (Fc region), on the strength of IgG antibody binding to a bacterial surface. Our results suggest a mechanism, intermolecular cooperativity, to account for at least part of the binding advantage exhibited by HGAC 39.G3 over HGAC 39.G1, HGAC 39.G2b, or HGAC 39.G3-derived $F(ab')_2$ fragments. According to this concept, closely approximated IgG3 molecules bound to bacterial epitopes associate through noncovalent Fc-Fc interactions, increasing the stability of the Ag-binding interaction. Consistent with this model for better IgG3 binding, relative to the IgG1 and IgG2b, are the preliminary observations that suggest that HGAC 39.G3 dissociates less rapidly from the bacteria than HGAC 39.G1 or HGAC 39.G2b.

We believe that optimal conditions for Fc-Fc association, which is likely to be concentration-dependent, occur at the Ag-binding interface, where the local concentration of antibody can approach high values. However, our mechanism does not exclude the possibility that Fc-Fc association might occur in solution (20).

Since IgG3 differs from IgG1 and IgG2b in the C_H1 and hinge regions, as well as in the Fc region (21), we cannot rule out a role for segmental flexibility in contributing to the binding differences observed among HGAC 39.G3, HGAC 39.G1, and HGAC 39.G2b. However, if as previously suggested (8), IgG3 is slightly more flexible than IgG1 and considerably less flexible than IgG2b, flexibility is not likely to be a key factor in causing the binding differences between HGAC 39.G3 and HGAC 39.G1 or HGAC 39.G2b, unless IgG3 possesses the optimum flexibility. The binding differences between HGAC 39.G2b and HGAC 39.G1 might be partially explained by the greater flexibility of murine IgG2b than IgG1 (8). Subclass-associated differences in segmental flexibility would not explain the ability of HGAC 39.G3 to engage in cooperative binding, although such differences might contribute to the inability of HGAC 39.G1 and HGAC 39.G2b to exhibit significant cooperativity. Alternatively, HGAC 39.G1 and HGAC 39.G2b may fail to exhibit cooperative binding because the $\gamma1$ and $\gamma2b$ Fc regions, respectively, do not self-associate with sufficient affinity.

These results, when considered from an evolutionary perspective, may provide insight into the preferential elicitation of murine IgG3 antibodies by bacterial polysaccharide Ag, including GAC (13, 14). Antibodies specific for bacterial polysaccharides generally exhibit low intrinsic affinities for these Ag (19). Therefore, it is conceivable that the ability of the IgG3 molecules to engage in cooperative binding represents an adaptation that facilitates antibody binding to bacterial surfaces, resulting in more efficient bacterial clearance. Indeed, we have found that the increased binding to GAiV of HGAC 39.G3, relative to HGAC 39.G1 and HGAC 39.G2b, is associated with greater uptake of the first component of complement (L. J. N. Cooper, manuscript in preparation). Additional experiments are in progress to evaluate IgG subclass-associated differences in functions associated with bacterial immunity.

If the ability to engage in cooperative binding is a murine IgG3-associated adaptation, then IgG3 antibodies specific for epitopes other than GlcNAc should exhibit cooperative binding and should bind more effectively than V domain-identical antibodies of other IgG subclasses. We have recently obtained evidence for IgG subclass-associated differences in binding and cooperative binding (IgG3 > IgG1) for two pairs of phosphorylcholine-specific mAb (IgG3 parent-IgG1 switch variant; L. J. N. Cooper, et al., manuscript in preparation). In a third system, we have observed differences in binding (IgG3 > IgG1) for a pair of *Pseudomonas aeruginosa* immunotype 1 LPS O-side chain-specific mAb (IgG3 parent-IgG1 switch variant; J. R. Schreiber et al., manuscript in preparation). Furthermore, on exposure to *P. aeruginosa*, the IgG3 anti-LPS mAb is substantially more effective than the IgG1 switch variant in activating complement and in mediating opsonophagocytosis (J. R. Schreiber et al., manuscript in preparation). These results suggest that the IgG subclass-associated differences in binding observed for the GAC-specific mAb are not a function of the particular Ag specificity of these mAb, and that IgG subclass-associated differences in binding are of more general significance.

In conclusion, our results may provide a physiologic context for the self-aggregating properties of murine IgG3 observed in a variety of studies (22–25). The in vivo relevance of our observations is also supported by evidence for the occurrence of IgG3 Fc-Fc interactions in mice suffering from IgG3-mediated tissue injury (26). Finally, given that mammalian and pathogen-derived carbohydrates sometimes share antigenic components (27), perhaps the cooperative binding mechanism, in conjunction with the typically low intrinsic affinities of polysaccharide-specific antibodies, represents a mechanism for self-nonself discrimination based on differences in

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epitope spacing as well as differences in epitope structure.

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