0022-1767/93/15012-5400$02.00/0
The Journal of Immunology

Vol 150, 5400–5407, No. 12, June 15, 199
Printed in U.S.A.

Studies on Antigen Binding by Intact and Hinge-Deleted Chimeric Antibodies[1]

Carol Horgan,[2] Kathy Brown, and Seth H. Pincus[3]

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840

Abstract. A matched set of chimeric IgG1 and IgG4 antibodies were used to investigate the role of the IgG hinge in binding to Ag with differing space between the epitopes. Antibodies bearing identical V regions and either IgG1 or IgG4 C regions were engineered with and without hinges. We measured the binding of these antibodies to the peptide CYYYEEEEY and to CYYYEEEEY-BSA conjugates with decreasing numbers of peptides per BSA molecule. We earlier showed that V region differences in antibodies could affect Ag binding patterns in solid-phase but not solution-phase assays; however, both types of assay yielded similar results for the hinge-deleted antibodies. Binding of CYYYEEEEY-BSA by hinge-deleted and intact IgG1 was similar, but intact IgG1 bound free peptide better than did hinge-deleted IgG1. Intact IgG4 antibody bound less well to CYYYEEEEY and CYYYEEEEY-BSA than did IgG1 but, surprisingly, hinge-deleted IgG4 showed better binding than did intact IgG4 and was more like the IgG1 antibodies in binding affinity. Thus, the IgG4 hinge may impart a structural constraint that prevents high affinity binding to Ag. The hinge-deleted IgG4 antibody did not activate C, although it bound Ag similarly to IgG1. This study is the first to address the effect of the IgG hinge on Ag binding by using well defined Ag with different epitope densities. Our results may provide an explanation for the apparent low affinity of IgG4 antibodies. *Journal of Immunology,* 1993, 150: 5400.

The use of matched sets of chimeric antibodies, i.e., identical V regions paired with C regions of different classes or subclasses, has been instrumental in the investigation of human Ig effector functions (1). Generally, binding of chimeric antibody to its epitope is thought to be unaffected by its subclass. In some cases, however, subclass differences do alter Ag binding specificity (2–4). Also, antibodies with mutations in the C region were shown to have increased Ag binding affinity, compared with wild-type antibody (5–7).

The C region domains of IgG are responsible for the effector functions of the antibody. Within the C region, the hinge is the least conserved region between human IgG subclasses. The role of the IgG hinge is to provide inter-H chain stability through disulfide linkages and also to provide segmental flexibility of the antibody (8–10). Additionally, the hinge may have a role in C activation, because hinge-deleted derivatives of C-fixing IgG subclasses do not activate C (8, 10–12). As yet, this role is not completely understood.

In previous reports, we showed that V region differences affected Ag binding and C activation by immobilized immune complexes (13, 14). As the epitope density decreased the binding to Ag differed slightly but C activation differed significantly. This may have been the result of either differences in the fit of the Ag into the Ag binding site or a change in flexibility caused by the V region. Because the hinge is responsible for segmental flexibility, we wanted to determine whether deletion of the hinge region would result in differences in Ag binding similar to those observed with V region alterations. We therefore examined the role of the hinge in the binding of antibodies to Ag with increasing distance between epitopes. Hinge-deleted variants of IgG1 and IgG4 chimeric antibodies that have identical V regions were prepared and used in solid-phase and solution-phase

Received for publication July 29, 1992. Accepted for publication March 22, 1993.

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[1] This work was funded in part by an Arthritis Investigator Award to C. H.

[2] Present address: Department of Molecular Medicine, University of Auckland School of Medicine, Auckland, New Zealand.

[3] Address correspondence and reprint requests to Dr. Seth H. Pincus, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, MT 59840.

[4] Abbreviations used: PEG, polyethylene glycol.

...noassays to measure the binding to Ag. The results ...ed that hinge-deleted IgG1 was not significantly dif... from intact IgG1 but did show some differences at ...epitope density. More interestingly, hinge-deleted IgG4 ...d Ag better than did intact IgG4, regardless of epitope ...ity.

...terials and Methods

...bodies and Ag

...meric antibodies were expressed from murine V_H genes ...ed to human IgG1 or IgG4 C_H genes, in the κ-produc... cell line T17.2, as described previously (14). A sche... diagram of the H chain constructs is shown in Figure ... Hinge-deleted IgG1 C_H genes were generated by PCR[4] ...nology. Briefly, the C_H1 exon (including flanking ...ence) and the C_H2 plus C_H3 exons with their flanking ...ons of introns were amplified by PCR. The primers ...the C_H1 region contained a 5′ *Eco*RI site and a 3′ ...I site, as follows: upstream primer, 5′-TAGGAAT-...CCAGCTCTGTCCCACACC-3′; downstream primer, ...TAGGGTACCTTTGGGGTGGGCTTAGGT-3′. The ...ers for the C_H2 plus C_H3 region contained a 5′ *Kpn*I ...and a 3′ *Eco*RI site, as follows: upstream primer, 5′-...GGGTACCAGGCCTCGCCCTCCAGCT-3′; down... primer, 5′-TAGGAATTCCGGGATGCGTCCA-...CAGG-3′. The two pieces were ligated together at the ...I site and then ligated into pSV-E-neo (15) at the *Eco*RI ... The V region genes were inserted at the *Bam*HI site as ...ribed (14). The assembly is shown schematically in ...ure 1*B*. Hinge-deleted IgG4 C_H genes were engineered ...site-directed mutagenesis. The IgG4 C region gene has ...nique *Pst*I site immediately upstream from the hinge ..., and we inserted a *Pst*I site downstream from the ...ge by using the Mutagene kit (Bio-Rad, Richmond CA) ... the mutagenic oligonucleotide 5′-TTGAGCT-...AGGGCGAGGCC-3′. The hinge exon was removed ...PstI digestion. For all H chain constructs, the same V_H ...e was ligated with the C_H genes in pSV-E-neo and ex...ed in the κ-producing T17.2 cell line (15). The anti...ies recognize the glutamic acid and tyrosine residues of ...ynthetic polypeptide (Tyr,Glu)-Ala-Lys (16).

...ptide CYYYEEEEY was synthesized by Dr. John ...gan, National Institutes of Health Biological Resources ...ch, and Dr. Jean Starkey, Montana State University ...eman MT). Maleimated BSA was purchased from ...e Laboratories (Rockford, IL), and the peptide was ...gated to it (via the amino-terminal cysteine) at various ...de to BSA ratios as described (13). The peptide to BSA ...ratios of the conjugates were determined by the size ...e conjugate according to SDS-PAGE.

...viations used in this paper: PCR, polymerase chain reaction; PEG, ...ylene glycol.

Sequencing

mRNA was prepared from cell cultures by using oligo-(dT)-cellulose (Beckton-Dickinson, Bedford, MA) (17). cDNA fragments of the V_H or C_H regions were prepared by using the Gene Amp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). The V region primers were 5′-CTGCAGCAGCCT-3′ (upstream primer) and 5′-TG-AGGAGACGGTGACCGT-3′ (downstream primer). The C region primers were 5′-GGCCCATCGGTCTTCCCC-CTGGC-3′ (upstream primer) and 5′-TAGGAATTCCCG-GAGACAGGGAGAG-3′ (downstream primer). Sequencing was performed with Sequenase version 2.0 (United States Biochemical Corp., Cleveland OH).

Solution-phase binding assays

Peptide alone and peptide-BSA were radioiodinated with ^{125}I by using Iodogen (Pierce). Binding of Ag in solution was determined by incubating ^{125}I-peptide or ^{125}I-peptide-BSA with various concentrations of antibody and then precipitating the complexes in 7.5% PEG 8000 (13). In the absence of antibody ≤5% of ^{125}I-labeled peptide and peptide-BSA were precipitated. Association constants were calculated as described (18).

Solid-phase binding assays

ELISA were used to measure the binding of antibody to immobilized Ag. Microtiter plates (Immunlon 2; Dynatech, Chantilly, VA) were coated with the peptide-BSA conjugates at 1 μg/ml and then blocked with 1% BSA in PBS. Antibodies (at various concentrations) were incubated with the immobilized Ag for 1 h at 37°C and washed. Bound antibody was detected with alkaline phosphatase-conjugated anti-κ antibody or alkaline phosphatase-conjugated IgG1 or IgG4 antibody (13, 14). Controls included irrelevant primary antibodies or maleimated BSA (without peptide) as Ag, and these gave readings equal to background ($OD_{405} \leq 0.02$). Measurements were made in duplicate and the OD values were averaged. The difference between duplicate measurements was <10%. For each experiment, all antibodies were tested simultaneously on the same plate, so plate to plate variation was eliminated.

C activation

C1q binding by the antibodies in immobilized complexes was measured in the C activation ELISA. C activation in solution was measured by using an immune hemolysis assay. Both assays were described previously (13). Finally, antibodies (without Ag) were heat aggregated at concentrations of 100 μg/ml by incubation at 63°C for 30 min. These were tested for C activation by using the immune hemolysis assay.

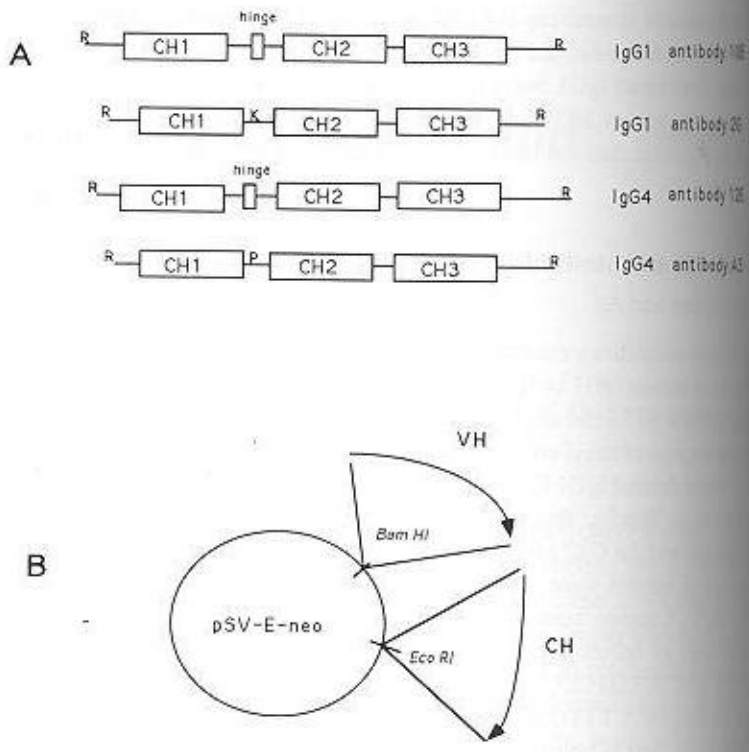

FIGURE 1. Construction of hinge-deleted antibodies. Human IgG1 and IgG4 H chain genes from genomic libraries were used to construct the chimeric H chain genes. The hinge exons were removed by PCR technology or site-directed mutagenesis. *A*, schematic diagram of the C region genes used to create the chimeric antibodies used in this study. *R*, *Eco*RI site; *K*, *Kpn*I site; *P*, *Pst*I site. *B*, the vector pSV-E-neo (gift of Dr. Thomas Simon) was used to assemble the C region genes with the rearranged murine V region gene T17 (27). The completed H chain genes were transfected into the κ-producing cell line T17.2, as described previously.

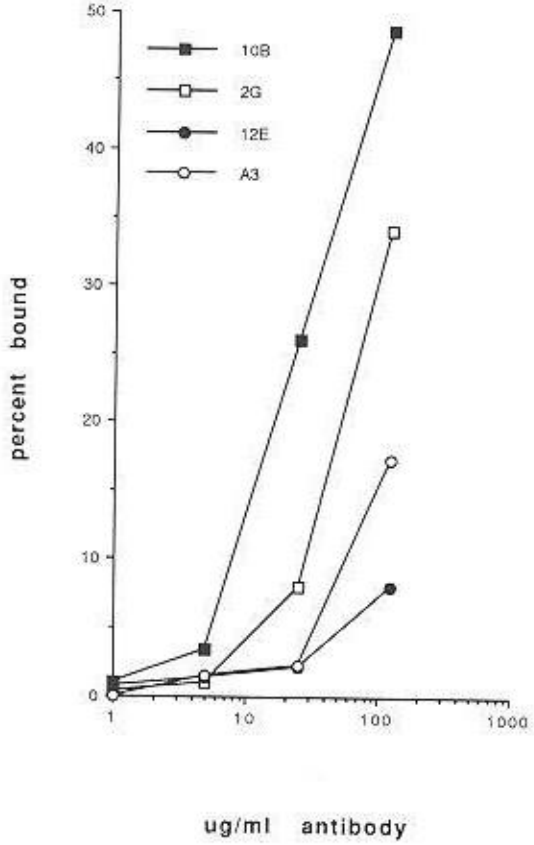

FIGURE 2. PEG precipitation of ^{125}I-labeled CYYYEEEEY by intact and hinge-deleted antibodies. ^{125}I-labeled peptide CYYYEEEEY at 1 μg/ml was mixed with the antibodies at increasing antibody concentration and then the mixtures were precipitated in 7.5% PEG. The percentage of ^{125}I cpm precipitated is shown on the *y*-axis. The data shown are the averages of at least two separate experiments.

Results

Antibodies

The antibodies used in this study were raised against the synthetic Ag (Tyr,Glu)-Ala-Lys and recognize the tyrosine and glutamic acid residues (16). Antibodies 10B and 2G are IgG1 chimeras with and without a hinge region, respectively (Fig. 1). Antibodies 12E and A3 are IgG4 chimeras with and without a hinge, respectively. We determined from cDNA sequencing that all four antibodies have identical V_H regions, and there are no differences in the C_H regions from the expected sequences for IgG1 or IgG4 except for the loss of the hinge regions in 2G and A3. All antibodies have the same murine κ L chains. Despite the lack of inter-H chain disulfide bonds, the hinge-deleted antibodies maintain the H_2L_2 configuration through noncovalent bonds. They have molecular masses of ≥150 kDa, as determined by gel exclusion chromatography and PAGE, and migrate to 7S in a sucrose density gradient (data not shown). Additionally, all four antibodies used in this study precipitated equally in 7.5% PEG (data not shown).

Binding of free peptide

We previously tested the binding of antibody 10B to a series of linear peptides consisting of tyrosine and glutamic acid residues and found that it bound to the peptide YYYEEEEY. That peptide was prepared with a carboxyl-terminal cysteine conjugated to BSA. The binding to CYYYEEEEY-BSA by 10B has been reported (13). Here

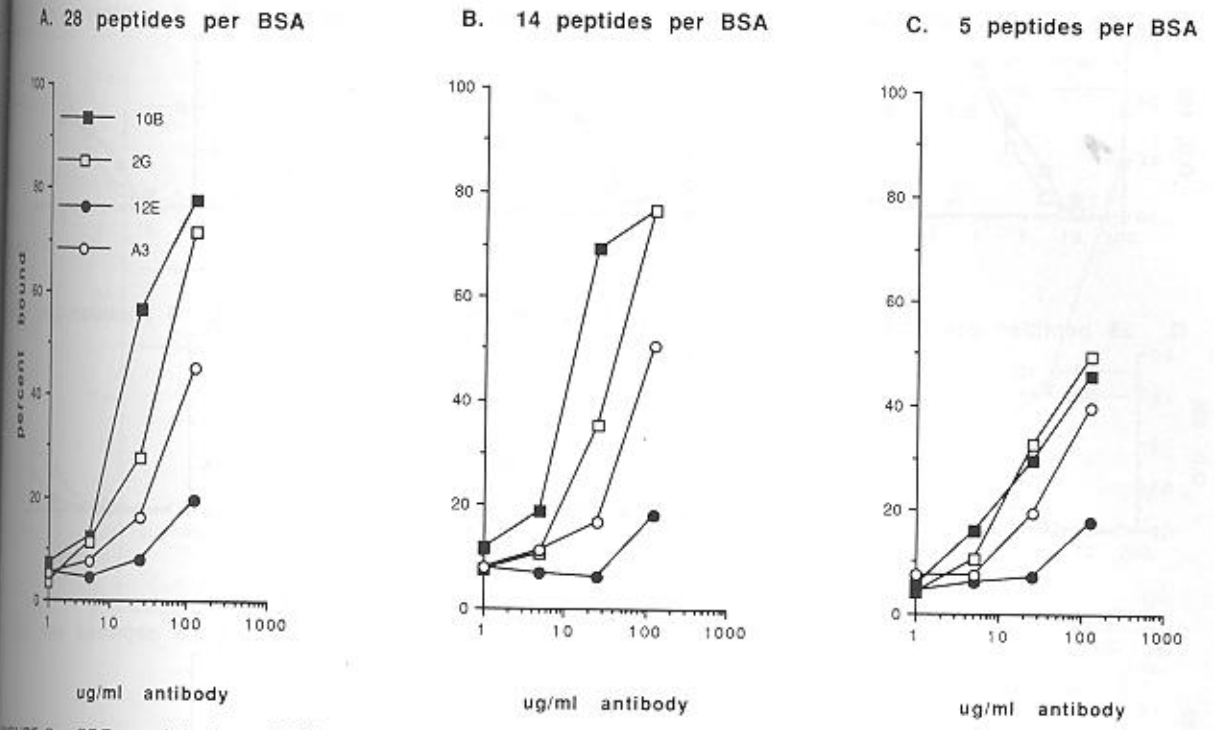

FIGURE 3. PEG precipitation of ^{125}I-CYYYEEEEY-BSA conjugates. ^{125}I-labeled CYYYEEEEY-BSA at 5 μg/ml (based on BSA content) was mixed with the antibodies and precipitated as in Figure 2. *A*, CYYYEEEEY-BSA at a peptide:BSA ratio of 28:1 was precipitated with increasing amounts of the IgG1 antibodies 10B (intact) and 2G (hinge deleted) or the IgG4 antibodies 12E (intact) and A3 (hinge deleted). *B*, CYYYEEEEY-BSA at 14 peptides/BSA was precipitated. *C*, CYYYEEEEY-BSA at 5 peptides/BSA was precipitated.

We used antibodies with the same V region to examine the effect of the hinge on Ag binding by IgG1 and IgG4 antibodies. The binding of ^{125}I-labeled CYYYEEEEY by the intact and hinge-deleted antibodies was measured in solution with the PEG assay. To calculate the equilibrium association constant, the antibody concentration was held at 100 μg/ml and the ^{125}I-CYYYEEEEY concentration was varied. To measure directly the amount of peptide bound by a particular amount of antibody, we held the ^{125}I-CYYYEEEEY concentration constant and increased the antibody concentration by degrees. In this latter assay, antibody 10B (intact IgG1) bound and precipitated CYYYEEEEY better than did antibody 2G (hinge-deleted IgG1) but the differences diminished at high antibody concentration (Fig. 2). Despite this apparent difference in relative affinity, the association constants of 10B and B11 for peptide were not significantly different (1.00×10^4 M^{-1} and 2.54×10^4 M^{-1}, respectively). The IgG4 antibodies 12E (intact IgG4) and A3 (hinge deleted) precipitated the peptide only at the highest antibody concentration, but antibody A3 precipitated more peptide than did antibody 12E (Fig. 2). Still, the association constants for the IgG4 antibodies 12E and A3 were not significantly different from each other or from the IgG1 antibodies (2.61×10^4 M^{-1} and 1.54×10^4 M^{-1}, respectively, for 12E and A3).

Binding of peptide-BSA conjugates

The peptides were conjugated to BSA via the amino-terminal cysteine by using a maleimide linker, at peptide to BSA molar ratios of 28:1, 14:1, and 5:1. The conjugates were iodinated with ^{125}I and used in the PEG assay with the antibodies. Figure 3 shows the PEG precipitation of the CYYYEEEEY-BSA conjugates by the antibodies. At high epitope density of CYYYEEEEY-BSA, antibody 10B bound slightly better than did antibody 2G (Fig. 3, *A* and *B*). At a ratio of 5 peptides/BSA, intact and hinge-deleted IgG1 bound equally (Fig. 3*C*). At all CYYYEEEEY:BSA ratios the IgG4 antibody A3 (hinge-deleted) bound more Ag than did the intact IgG4 antibody 12E (Fig. 3).

The differences between intact and hinge-deleted antibodies were parallel, but less marked, when the binding was measured with a solid-phase ELISA. IgG1 antibodies 10B and 2G bound roughly equivalently to immobilized CYYYEEEEY-BSA at all peptide:BSA ratios, although consistantly more 10B was bound than 2G (Fig. 4, *A* to *C*). This was true for assays in which the secondary (detecting) antibody was alkaline phosphatase-conjugated anti-κ (Fig. 4, *A* to *C*) or alkaline phosphatase-conjugated anti-IgG1 (data not shown). The IgG4 antibodies differed slightly, depending on the secondary antibody used in the ELISA.

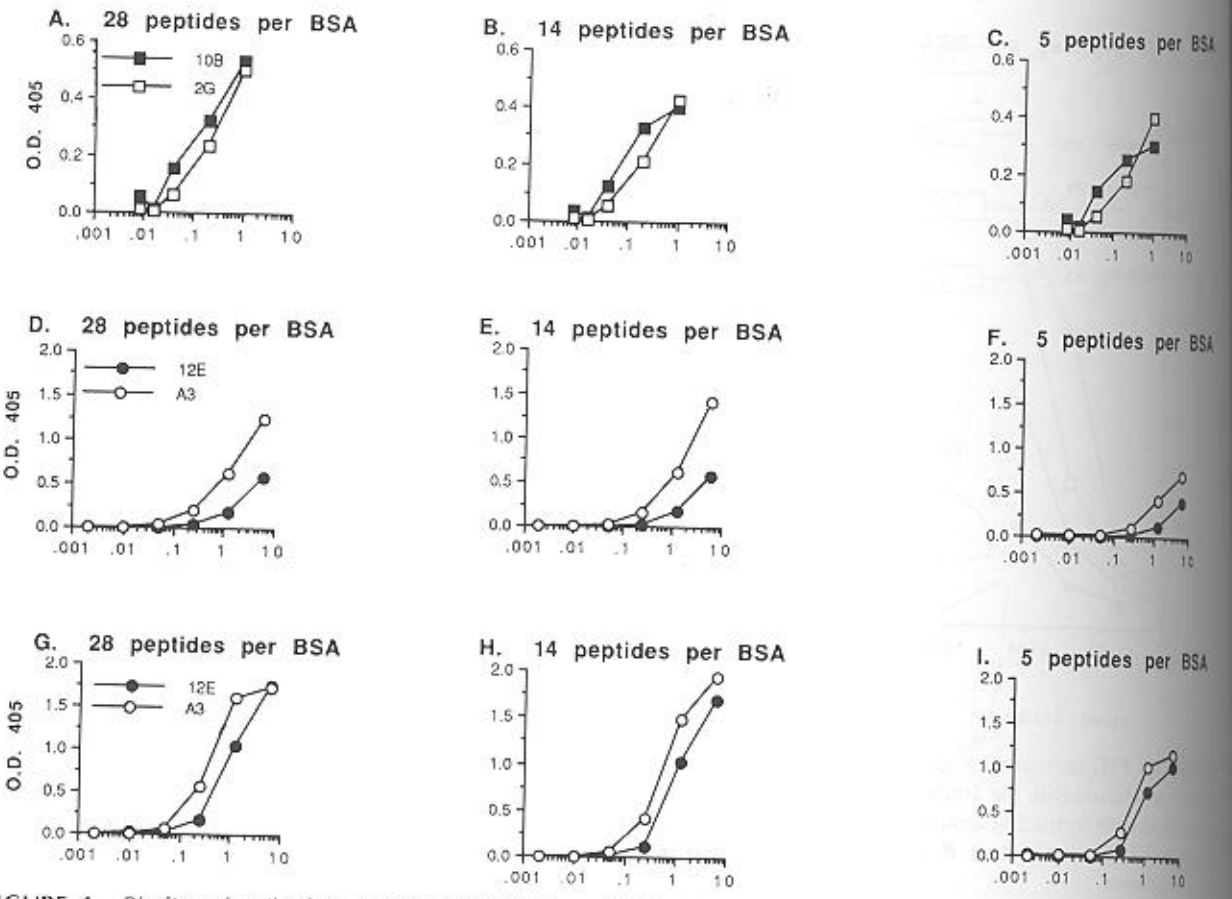

FIGURE 4. Binding of antibodies to CYYYEEEEY-BSA in an ELISA. Microtiter plates were coated with 1 μg/ml CYYYEEEEY-BSA (based on BSA content) at various peptide:BSA ratios. Antibodies were allowed to bind to the plates and were detected with alkaline phosphatase-conjugated anti-κ or alkaline phosphatase-conjugated anti-IgG4. *A* to *C*, binding of IgG1 antibodies 10B and 2G to CYYYEEEEY-BSA with peptide:BSA ratios of 28:1, 14:1, and 5:1, respectively, detected with anti-κ second antibody. *D* to *F*, binding of IgG4 antibodies 12E and A3, detected with anti-κ second antibody. *G* to *I*, binding of IgG4 antibodies 12E and A3, detected with anti-IgG4 second antibody.

The binding signal was lower with anti-κ (Fig. 4, *D* to *F*), compared with anti-IgG4 (Fig. 4, *G* to *I*), as the detecting antibody. However, with either secondary antibody more A3 was bound than 12E.

C activation

The binding of C1q by antibody 10B bound to CYYYEEEEY-BSA in the C activation ELISA has been described (14). We used the same assay to measure the C1q binding of the matched set of antibodies. Under conditions where approximately equal amounts of antibody were bound by the Ag, only the intact IgG1 antibody activated C and bound C1q (Fig. 5*A*). C activation by antibody/CYYYEEEEY-BSA was also measured in solution by using an immune hemolysis assay. Of the four antibodies tested, only the intact IgG1 antibody activated C (Fig. 5*B*). Heat-aggregated IgG can activate C in the absence of specific Ag (19). Heat-aggregated 10B, but not heat-aggregated 2G, activated C in the immune hemolysis assay (data not shown).

Discussion

We used a matched set of chimeric mouse-human IgG1 and IgG4 antibodies to investigate the role of the IgG hinge in binding to Ag with epitopes at different densities. Previous studies have used matched sets of chimeric antibodies with natural or substituted hinges to investigate antibody effector function (1, 12, 20, 21), but this is the first examination of the Ag binding characteristics of hinge-deleted antibodies. The hinge region controls segmental flexibility of the antibody (8–10) and so removal of the hinge may or may not affect the binding of antibodies to Ag, depending on the distance between epitopes. To investigate this hypothesis, CYYYEEEEY-BSA conjugates with decreasing peptide:BSA molar ratios were used to identify potential binding differences between intact and hinge-deleted IgG1 and IgG4 antibodies. We found that removal of the IgG1 hinge resulted in slightly lower relative binding affinity, but removal of the IgG4 hinge increased the relative affinity, irrespective of epitope density.

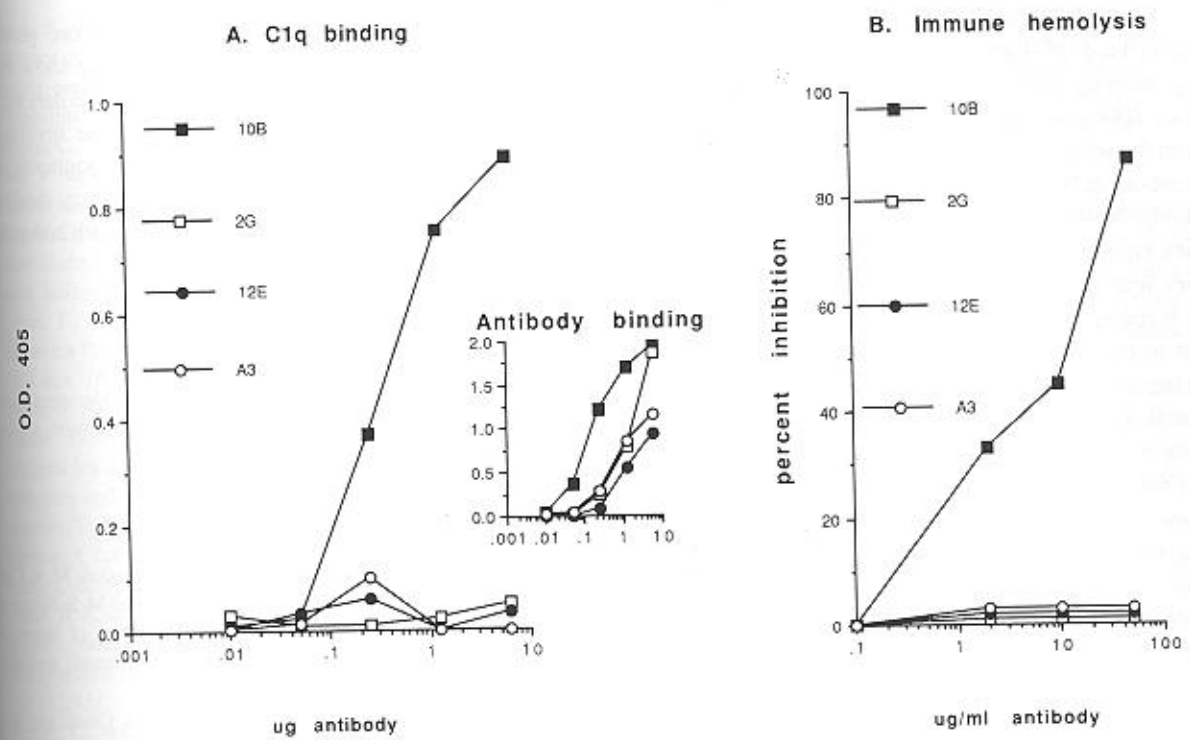

FIGURE 5. C activation by intact and hinge-deleted antibodies. *A*, C1q-binding ELISA. IgG1 and IgG4 antibodies were allowed to bind to immobilized CYYYEEEEY-BSA (28:1). Normal human serum was then added as a source of C, and after washing the C1q bound was detected with alkaline phosphatase-conjugated anti-C1q. *Inset*, the amount of antibody bound was measured with alkaline phosphatase-conjugated anti-IgG1 or anti-IgG4. *B*, C activation by soluble immune complexes. CYYYEEEEY-BSA (28:1) was mixed with increasing amounts of antibody and tested for C activation in the immune hemolysis assay. The amount of C consumed by the immune complexes was measured as the percentage of inhibition of lysis of sensitized SRBC.

The binding affinity of antibody for Ag can be calculated by a variety of methods. We have found that the equilibrium association constant for a given antibody/peptide pair can vary considerably depending on the assay used (C. Hogan and S. Pincus, manuscript in preparation). Solution-phase binding of antibody to radiolabeled peptide and separation of bound vs free peptide is the most widely accepted process. By such an assay, we find no significant difference in the equilibrium association constants of any of the four antibodies assessed here. However, it is clear from Figures 2 and 3 that there exist differences in the amount of peptide bound per microgram of antibody at lower antibody concentrations. We describe these as differences in relative affinity. We previously showed that differences in relative binding could be apparent in solid-phase but not solution-phase assays (22). In this case, we observe the differences in both solution- and solid-phase assays. Assessment of affinity by using peptides or haptens provides a measurement of intrinsic affinity, whereas assessment with multivalent Ag provides a measurement of functional affinity. We note the same differences in relative affinity in assays that measure binding to peptide or peptide-BSA. Thus, our observation is independent of the valency of the Ag. (Both the intact and hinge-deleted antibodies are bivalent.)

The differences between the intact and hinge-deleted IgG1 antibodies (10B and 2G, respectively) were not remarkable. Slightly more Ag was precipitated by the intact antibody 10B than by the hinge-deleted antibody 2G (Figs. 2 and 3). The percentage of Ag precipitated by a given amount of either antibody decreased with fewer peptides per BSA, and unconjugated peptide was precipitated less well than peptide-BSA conjugates. Because 10B and 2G precipitated equally in 7.5% PEG, the decrease in percentage of precipitation of the ^{125}I-Ag reflects smaller latticed antibody-Ag complexes. Antibodies 10B and 2G bound immobilized Ag equivalently in the ELISA. We conclude that antibody 10B binds Ag with only slightly higher affinity, compared with antibody 2G. Under the conditions tested the differences were small; therefore, the lack of the hinge did not significantly affect Ag binding or Ag/antibody stoichiometry. Although the hinge-deleted IgG1 antibody 2G bound to the Ag and formed immune complexes similarly to antibody 10B, it did not activate C when either complexed with Ag or heat aggregated. This is in agreement with the results of others (8, 10–12). Therefore, the inability of hinge-deleted antibodies to activate C is not simply the result of the antibodies not forming immune complexes with the appropriate stiochiometry or lattice.

Surprisingly, an increase in binding was observed for the hinge-deleted IgG4 antibody A3, compared with the intact IgG4 antibody 12E. Antibody A3 bound free peptide and peptide-BSA conjugates better than did intact antibody 12E in both the solution- and solid-phase assays. We noted some differences in the IgG4 ELISA depending on the detecting antibody. Anti-κ and anti-IgG1 recognized the IgG1 antibodies equally (13), but the anti-κ bound the IgG4 antibodies less well than did the anti-IgG4 second antibody. This is probably because of the accessibility of the IgG4 L chain to the detecting antibody. We performed the assay with the anti-κ antibody so that we could compare the IgG1 antibodies with the IgG4 antibodies by using the same secondary antibody. Then, to determine whether the differences noted between the IgG4 antibodies with and without a hinge were only an artifact of the detecting antibody, we performed the assay again with a different (anti-IgG4) secondary antibody. For this reason, we have shown the data for both secondary antibodies. With either secondary antibody A3 bound better than 12E; thus, we believe that the result is accurate. Furthermore, the differences between intact and hinge-deleted IgG4 were exactly the same when a second peptide, CEYYEYEEY, and its BSA conjugates were used as Ag (data not shown). Thus, the phenomenon is not restricted to a particular interaction between the Ag binding site and CYYYEEEEY. A V region mutation could account for the discrepancy, but that possibility was ruled out with cDNA sequencing of 12E and A3 mRNA. These results suggest that an IgG4 antibody lacking a hinge is intermediate in Ag binding activity between IgG4 and IgG1, as measured by the immunoassays shown in Figures 2 to 4. Because the hinge of IgG4 is short and relatively inflexible (8, 10, 23), perhaps it influences the Ag binding activity and its removal allows a better fit of the Ag in the Ag combining site. Phillips et al. (24) suggested that antibodies undergo structural strains as the Fab arms close to small angles. Also, V region differences can affect binding of antibodies to immobilized surfaces (13), suggesting that structural constraints on the antibody bound to an immobilized surface could affect the Ag binding. A similar mechanism may affect IgG4 antibodies, even in solution, with a structural constraint imposed by the hinge. However, even the binding of the free peptide was improved by the removal of the IgG4 hinge (Fig. 1). Another explanation is that the hinge region may influence the interaction of the H chain with the L chain, which could affect the Ag combining site.

The IgG4 response may often give rise to low affinity antibodies (25, 26). In this study, removal of the IgG4 hinge resulted in consistently higher Ag binding (Figs. 2 to 4). The functions of IgG4 antibodies that bind Ag poorly are not fully understood, but our data suggest that the IgG4 hinge may influence those functions.

The results from this and previous studies on these chimeric antibodies (13, 14) challenge some basic assumptions of Ig structure and function. We earlier showed that the V region differences affect C activation, generally thought to be a C region function. Here we show that C region alterations influence Ag binding. These data suggest that interactions between domains may be involved in structural changes after Ag binding. Crystallographic studies of these antibodies are in progress and may shed some light on the interactions of the antibodies with and without hinges when they bind peptide.

Acknowledgments

We thank Susan Smaus for secretarial assistance. We also appreciated the critical comments of Drs. John Swanson, Richard Morrison, and David Dorward.

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