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EFFECT OF H CHAIN V REGION ON COMPLEMENT ACTIVATION BY IMMOBILIZED IMMUNE COMPLEXES[1]

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Activation of C by immune complexes (IC) in tissues and the inflammatory consequences are major determinants in the pathogenesis of many autoimmune disorders. To assess the factors involved in C activation by such IC, we examined the binding of C components by chimeric IgG1 antibodies bound to immobilized Ag. We previously reported that alterations in the H chain V regions can affect the binding of first component of C (C1q) and a major breakdown product of the third C component (C3b) when otherwise identical antibodies were bound to immobilized (Tyr, Glu)-Ala–Lys. To evaluate C activation of these antibodies in well defined IC, we utilized a 9-amino acid peptide conjugated to BSA as Ag. The peptide:BSA conjugate was bound similarly by the two IgG1 antibodies which differed mainly in the CDR3 regions, but also in 9 other amino acids in the H chain V region. When soluble IC were prepared with the two antibodies, they activated C similarly. However, C activation by solid phase Ag-antibody complexes differed; we found that antibody 10B bound more C1q and C3b than antibody B11 did, unless the Ag was present at high density on the plates. These data suggest that the variable region differences affect C activation by these antibody when they are bound to immobilized Ag. Furthermore, these results underscore the differences in C activation by the same antibody depending upon whether the IC are free in solution or immobilized.

The inflammatory consequences of C activation by immune complexes in tissues such as skin, vasculature, and renal glomeruli are major determinants in the pathogenesis of many autoimmune disorders (1–5). Many factors such as antibody avidity and the makeup of the Ag-antibody lattice influence C activation. C activation by immune complexed IgG occurs when a single molecule of C1q is bound by two appropriately spaced Fc regions of IgG. Whether or not this process requires a conformational change in the IgG, as occurs in IgM antibodies, is still subject to debate. The C1q-binding site of IgG is in the C_H2 domain and involves residues Glu-318, Lys-320, Lys-322 (6), and probably Tyr-296 of the opposite H chain (7).

We recently reported on two chimeric antibodies which bound the branched chain polypeptide (Tyr,Glu)-Ala–Lys (which we now abbreviate (Y,E)-A–K by using the single letter amino acid code) (8). We found that V region differences between chimeric mouse/human IgG antibody could affect C activation by these antibodies when they were bound to immobilized Ag. The difference was not due to affinity or avidity of binding. We postulated that because the antibodies had different V_H regions they could have recognized epitopes on (Y,E)-A–K differently, and this could influence their pattern of binding and therefore their potential to activate C. Alternatively, the two antibodies could exhibit different allosteric constraints upon binding the immobilized Ag, and these structural differences could dictate the variance in C1q binding. In the present study, we have ruled out the former explanation. By using a peptide Ag, we found both antibodies bound the peptide CYYYEEEEY with the same affinity. When this peptide was coupled to a BSA carrier and incubated with antibody, well defined immune complexes were formed. Our data demonstrated that even when the antibodies bound to identical sites on a known antigenic matrix, differences in C1q binding were seen. Additionally we demonstrated differential C1q binding of immune complexes depending upon whether they were soluble or immobilized. If the results of these studies on chimeric antibodies can be extrapolated to the function of autoantibodies in vivo, then the variable regions of autoantibodies may influence their pathogenic potential by mechanisms secondary to Ag binding.

MATERIALS AND METHODS

Antibodies and Ag

Antibodies 10B and B11 were expressed from murine V_H genes joined to human IgG1 C_H genes. The constructs were transfected into murine hybridoma cells expressing murine L chain (8). The antibodies were directed against the glutamic acid (E) and tyrosine (Y) residues of the synthetic polypeptide (Y,E)-A–K (9). The antibodies differ only in the V_H region and have the same human C_H region and identical murine κ L chains. The V_H region differences are shown in Figure 1 (10). Peptide CYYYEEEEY was synthesized by Dr. John Coligan of the National Institutes of Health Biological Resources Branch. Maleimated BSA was purchased from Pierce Laboratories (Rockford, IL), and the peptides were conjugated to it at various peptide:BSA ratios. Unincorporated peptide was removed by desalting the conjugates on G-50 spin columns (Boeringher Mannheim, Indianapolis, IN). The molar ratio of peptide to BSA in the conjugate

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[3] Abbreviations used in this paper: C1q, first component of C; C3b, major breakdown product of the third C component; C_H, H chain constant region; PEG, polyethylene glycol; V_H, H chain V region; (Y,E)-A–K, the branched chain polypeptide (Tyr,Glu)-Ala–Lys.

Figure 1. Amino acid sequence of V_H region. The deduced amino acid sequence of the V_H regions of antibodies 10B and B11 were derived from the nucleotide sequences of the original V_H genes (10). These genes were used to construct the chimeric H chain genes (8). After these genetic manipulations, we confirmed the sequence and found no mutations. *Dashes* indicate identity. The sequences of the H chain constant regions and the entire L chains of the two antibodies do not differ.

```
                                   CDR1                                CDR2
    1           11          21          31          41          51          61
10B LQQPGAELVK PGASVKLSCK ASGYTFTSYW MHWVKQRPGR GLEWIGRIDP NSGGTKTNQK FKSKAT
B11 ---------- ---------- -------N-- I--------- ---------- ---A------ --N----

                                                CDR3
                                          D           J
   71          81          91          101         110
10B KPSSTAYIQL SSLTSEDSAV YYCARREVDG YFDVWGAGTT VTVSS
B11 ---N---N-- -----D---- -----GYDYS -TANDYW-QG TS-TVSS
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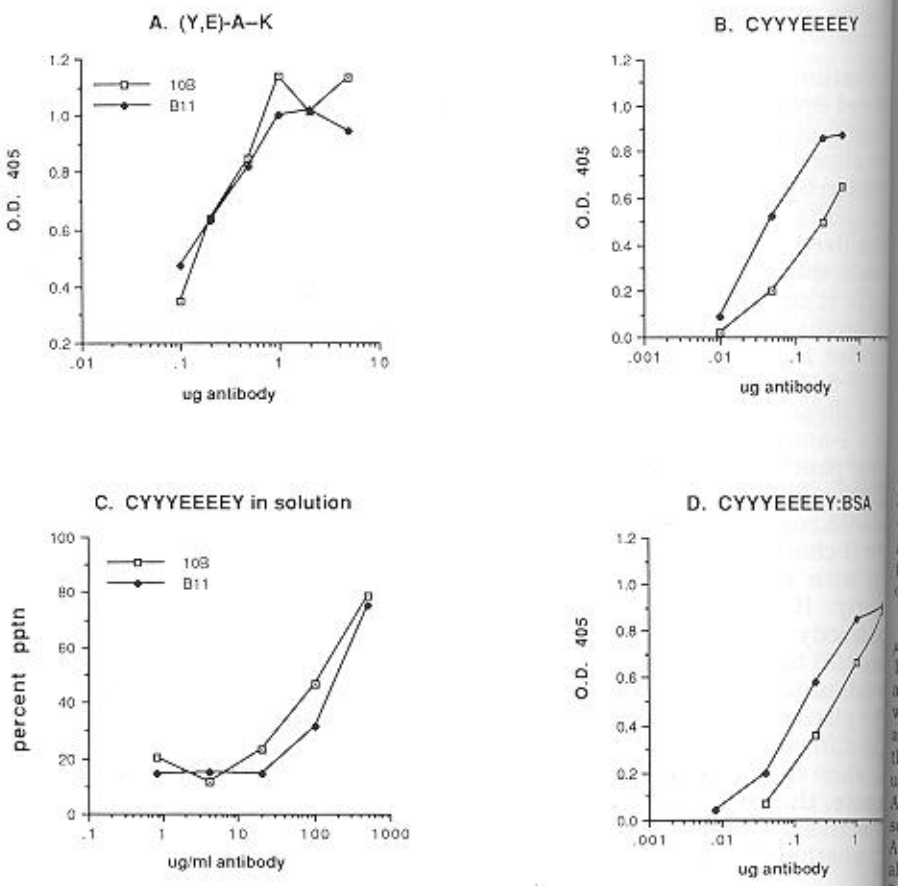


Figure 2. Binding of antibodies 10B and B11 to immobilized Ag. *A*, *B*, and *D*, Solid phase assays. Microtiter plates were coated with Ag and then reacted with increasing amounts of antibody. Bound antibody was detected with alkaline phosphatase-conjugated anti-κ antibody. *C*, Solution phase assay. ^{125}I-CYYYEEEEY at 5 μg/ml was mixed with increasing amounts of antibody, and the resulting complex was precipitated by PEG. In *A*, plates were coated with 10 μg/ml (Y,E)-A–K. In *B*, plates were coated with unconjugated peptide CYYYEEEEY at 10 μg/ml. In *C*, percent precipitation of peptide CYYYEEEEY. In *D*, plates were coated with BSA-conjugated peptide (CYYYEEEEY:BSA) at 5 μg/ml.

was determined by one or both of two methods. For some conjugates, the peptide was labeled with ^{125}I and the amount of peptides conjugated to BSA was determined from the counts/min taken up by BSA. The molar ratios were confirmed by determining the m.w. of the conjugate by SDS-PAGE. For the remaining conjugates, the peptide:BSA ratio was determined by comparing their m.w. to those of the first series of conjugates.

Evaluation of Antibody Binding to Ag

Solution assays. Peptide alone and peptide:BSA were radioiodinated with ^{125}I by using Iodogen (Pierce). Binding of unconjugated peptide in solution was determined by incubating ^{125}I-peptide with antibody and then precipitating the complexes in 7.5% PEG 8000 (11). Sucrose density gradients were employed to determine the size of immune complexes. ^{125}I-peptide:BSA was incubated with either antibody 10B or B11 at various Ag:antibody ratios for 1 h at 37°C. That mixture was then applied to an isokinetic sucrose density gradient, prepared according to the method of Johns and Stanworth (12). The gradients were centrifuged at 33,000 rpm until $\omega^2 t = 8.61 \times 10^{11}$. After centrifugation, 500-μl aliquots were successively removed from the top, and the fractions were counted in a gamma counter (Beckman Instruments, Redmond, WA). To examine the stoichiometry of the peptide:BSA/antibody complexes in solution, the ^{125}I-peptide:BSA was incubated with antibody at different Ag:antibody ratios as above, and then precipitated with an equal volume of 15% PEG 8000 or saturated ammonium sulfate (Baker Chemical Co., Phillipsburg, NJ). The percent ^{125}I-peptide:BSA precipitated was determined after spinning the samples for 5 min at 8,000 $\times$ *g* at 4°C.

Solid phase assays. ELISAs were employed to measure the binding of antibody to immobilized Ag. Microtiter plates (Immunl Dynatech, Chantilly, VA) were coated with peptide alone, or tide:BSA conjugates at various concentrations and then blocke 1% BSA in PBS. Antibody (at various concentrations) was inc on the immobilized Ag for 1 h at 37°C and washed. Bound an was detected with alkaline phosphatase-conjugated anti-κ an (8). When irrelevant mAb were assayed on the plates, there binding. Also, when maliemated BSA alone was used as immob Ag, there was no binding of antibodies 10B and B11. Measure were always made in duplicate and the absorbance values averaged. In all cases, there was less than 10% difference be duplicate measurements.

C Activation

C activation by soluble IC was examined in an immune he assay (13). Soluble IC were prepared as described above for th assays and these or buffer alone were incubated with an volume of normal human serum (diluted 1/10 in gelatin v buffer (13) for 20 min at 37°C. This mix was added to 25 sensitized SRBC (1.5% in GVB^{++}). The volume was brought ml with GVB^{++} and incubated 40 min at 37°C, then centri From the OD_{514} of the supernatants we calculated the perc inhibition of lysis. CYYYEEEEY:BSA or antibody alone (at centrations) did not inhibit lysis more than 15%. C1q bind soluble IC was measured in a solid phase ELISA as follows. Mi plates were coated with purified C1q (Cytotech, San Diego, C

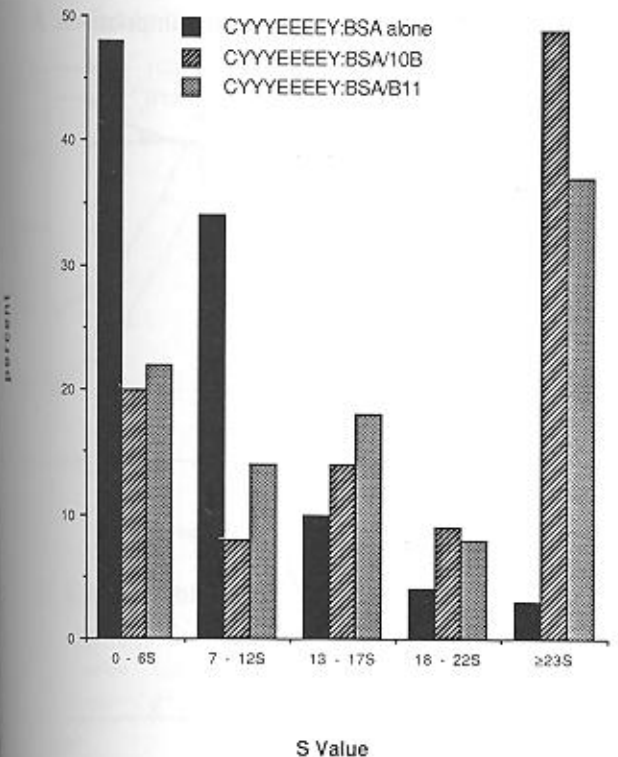


Figure 3. Sucrose density gradient sizing of soluble immune complexes. Soluble immune complexes were formed with ^{125}I-CYYYEEEEY:BSA at 2 μg/ml and either antibody at 100 μg/ml. BSA (1%) was present as carrier. The gradients were centrifuged and fractionated as described in *Materials and Methods*. The percent of total counts/min in each of the size groups is shown for ^{125}I-CYYYEEEEY:BSA alone or complexed with either antibody.

μg/ml then blocked with 1% BSA. Soluble IC were prepared with 100 μg/ml antibody and increasing amounts of CYYYEEEEY:BSA, and incubated on the plates for 1 h at 37°C and washed. Bound IC were detected with alkaline phosphatase anti-κ antibody. When antibody alone (without CYYYEEEEY:BSA) was used as a control, there was no binding of the second antibody. For immobilized IC, we used the C activation ELISA. Antibodies were bound to immobilized Ag and then incubated with normal human serum (1:50 in PBS supplemented with 1% BSA, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$). After 30 min at 37°C, the plates were washed and developed with alkaline phosphatase-conjugated anti-human C1q (The Binding Site, San Diego, CA) or anti-human C3d, which also recognized C3b (received from Ronald P. Taylor, University of Virginia). Control wells in which PBS (+1% BSA) was substituted either for normal human serum, antibodies 10B and B11, or both, always gave values less than or equal to background. The amount of antibody bound to immobilized peptide:BSA (measured by anti-κ second antibody) was not altered by the incubation with normal human serum.

RESULTS

Ag binding. We measured the binding of antibodies 10B and B11 to immobilized (Y,E)-A–K, peptide CYYYEEEEY, and CYYYEEEEY:BSA. (Y,E)-A–K was bound by both antibodies equivalently in an ELISA (Fig. 2A). The immobilized peptide was bound by antibody B11 better than antibody 10B (Fig. 2*B*). In solution, the peptide was bound somewhat better by antibody 10B as measured by PEG precipitation of the Ag/antibody complexes (Fig. 2*C*). To prepare a multivalent Ag, we conjugated the peptide to maliemated BSA via the N-terminal C which, in addition to providing the SH group for N-terminal attachment, also acted as a spacer so the sequence would be accessible to the antibodies. Unless we specifically state otherwise, we used a CYYYEEEEY:BSA conjugate with a molar ratio of 26 peptides per BSA in the subsequent studies. This ratio was determined by uptake of ^{125}I-peptide during the conjugation step and was confirmed by the m.w. of the conjugate by SDS-PAGE. When the conjugate CYYYEEEEY:BSA was immobilized onto microtiter plates both antibodies bound, and like the binding to the immobilized, unconjugated peptide, the binding of B11 to CYYYEEEEY:BSA was higher than that of 10B at lower amounts of antibody ($p < 0.001$ at 0.1 μg antibody input; $p < 0.01$ at 1 μg antibody input; and not significantly different at 5 μg input: $p > 0.05$) (Fig. 2*D*).

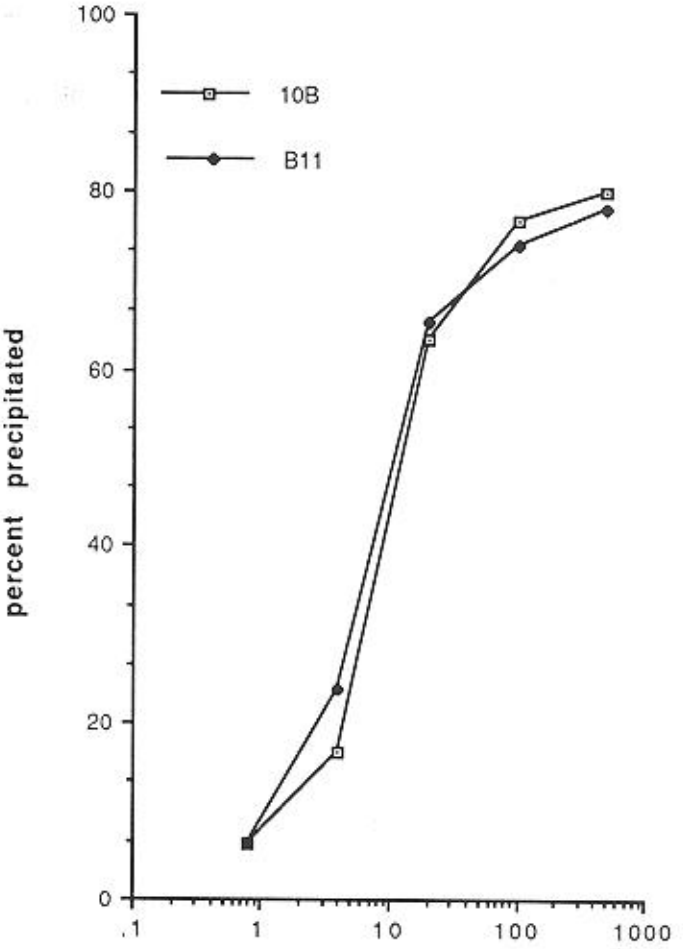


Figure 4. PEG precipitation of soluble ^{125}I-CYYYEEEEY:BSA/antibody immune complexes. Soluble immune complexes were formed with ^{125}I-CYYYEEEEY:BSA at 1 μg/ml Ag with increasing concentrations of antibody 10B and B11. BSA (1%) was present as carrier. The mixtures were precipitated in 7.5% PEG and the pellets counted. The percent of ^{125}I-Ag precipitated (y-axis) represents the average of triplicate measurements.

Determination of CYYYEEEEY:BSA/antibody stoichiometry. To examine the size of immune complexes formed with peptide-BSA conjugates and the two antibodies, sucrose density gradients were run on mixtures of ^{125}I-labeled CYYYEEEEY:BSA and either antibody. Twenty-four fractions corresponding to the S value (12) were collected and counted in a gamma counter. The fractions were grouped into size ranges of 0 to 6S, 7 to 12S, 13 to 17S, 18 to 22S, and ≥23S. The first two groups represent unbound Ag and antibody$_1$:Ag$_1$, respectively, based on the S values of the conjugate (5–6S) and anti-

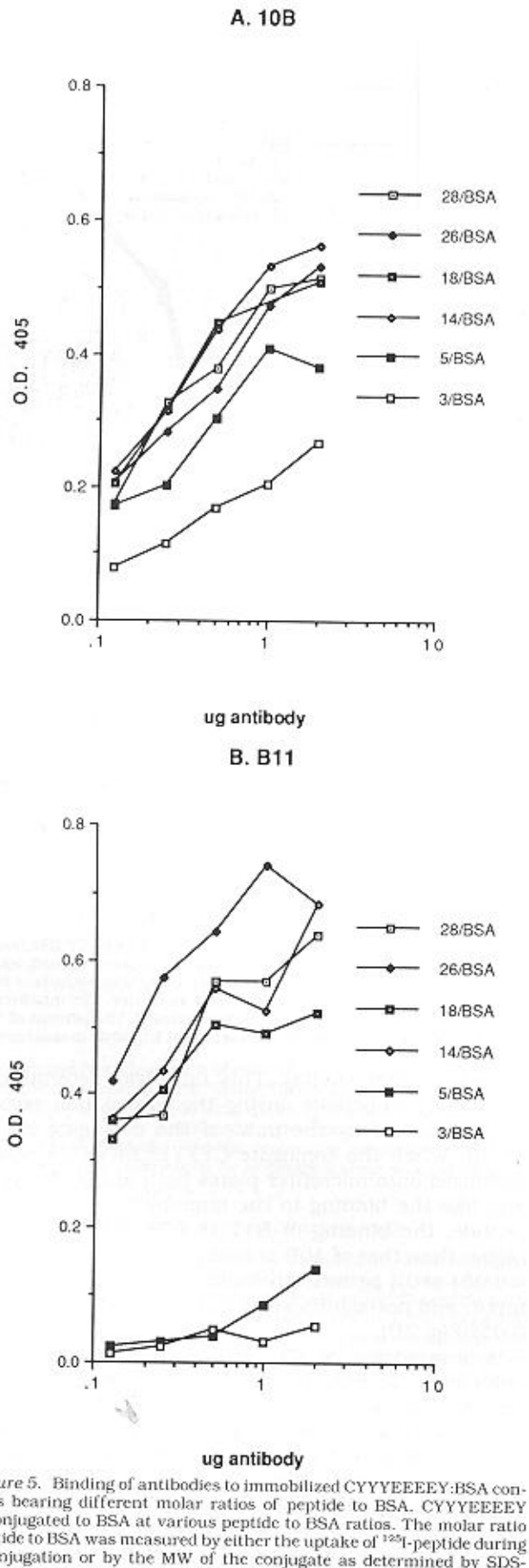
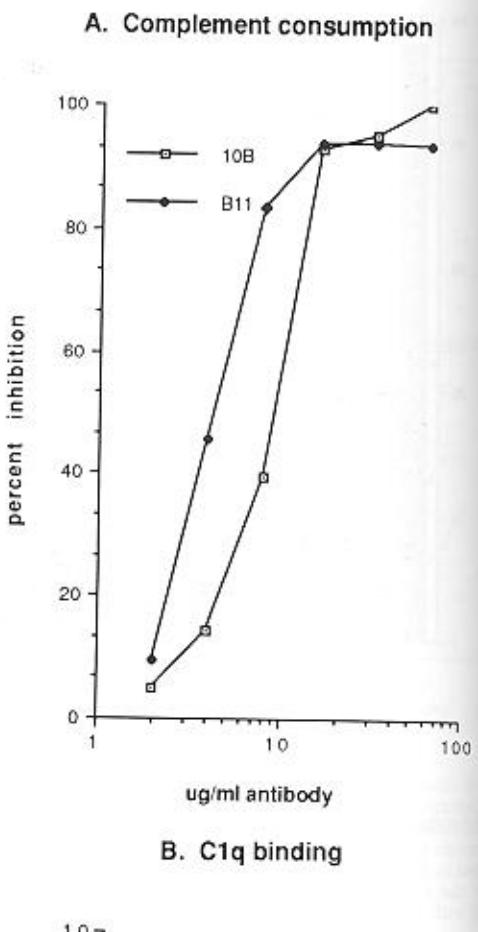


Figure 5. Binding of antibodies to immobilized CYYYEEEEY:BSA conjugates bearing different molar ratios of peptide to BSA. CYYYEEEEY was conjugated to BSA at various peptide to BSA ratios. The molar ratio of peptide to BSA was measured by either the uptake of ^{125}I-peptide during the conjugation or by the MW of the conjugate as determined by SDS-PAGE or both. The conjugates were coated onto microtiter plates at 1 μg/ml, then reacted with antibody as in other ELISA. *A*, Binding of antibody 10B to the conjugates as noted in the figure; *B*, binding of antibody B11.



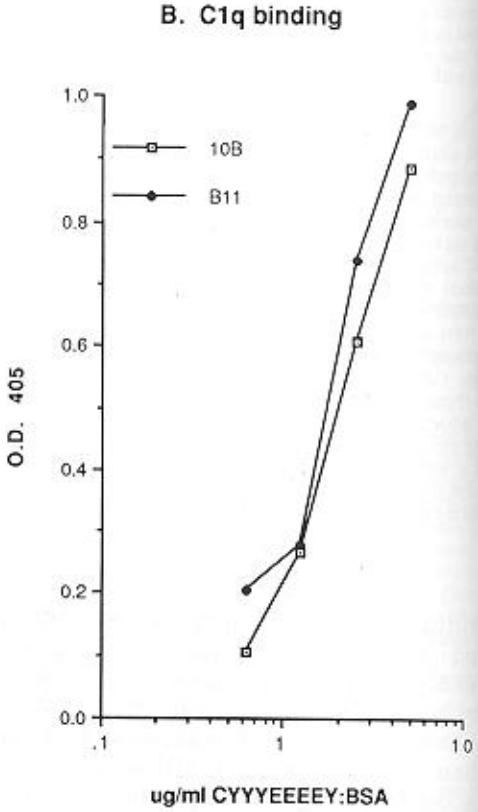


Figure 6. C activation and C1q binding by soluble CYYYEEEEY antibody complexes. *A*, Soluble complexes were formed with 5 CYYYEEEEY:BSA (26 peptides per BSA) and increasing amounts tibody. These were tested for C activation in an immune hemolysis as described in *Materials and Methods*. The amount of C consume the IC is measured by the percent inhibition of lysis of sensitized *B*, C1q binding of IC was measured in a solid phase ELISA. IC were f with 100 μg/ml antibody and increasing amounts of CYYYEEEEY (26 peptides per BSA). These were incubated on microtiter plated with 5 μg/ml purified C1q. Binding of complexes was detected with x second antibody.

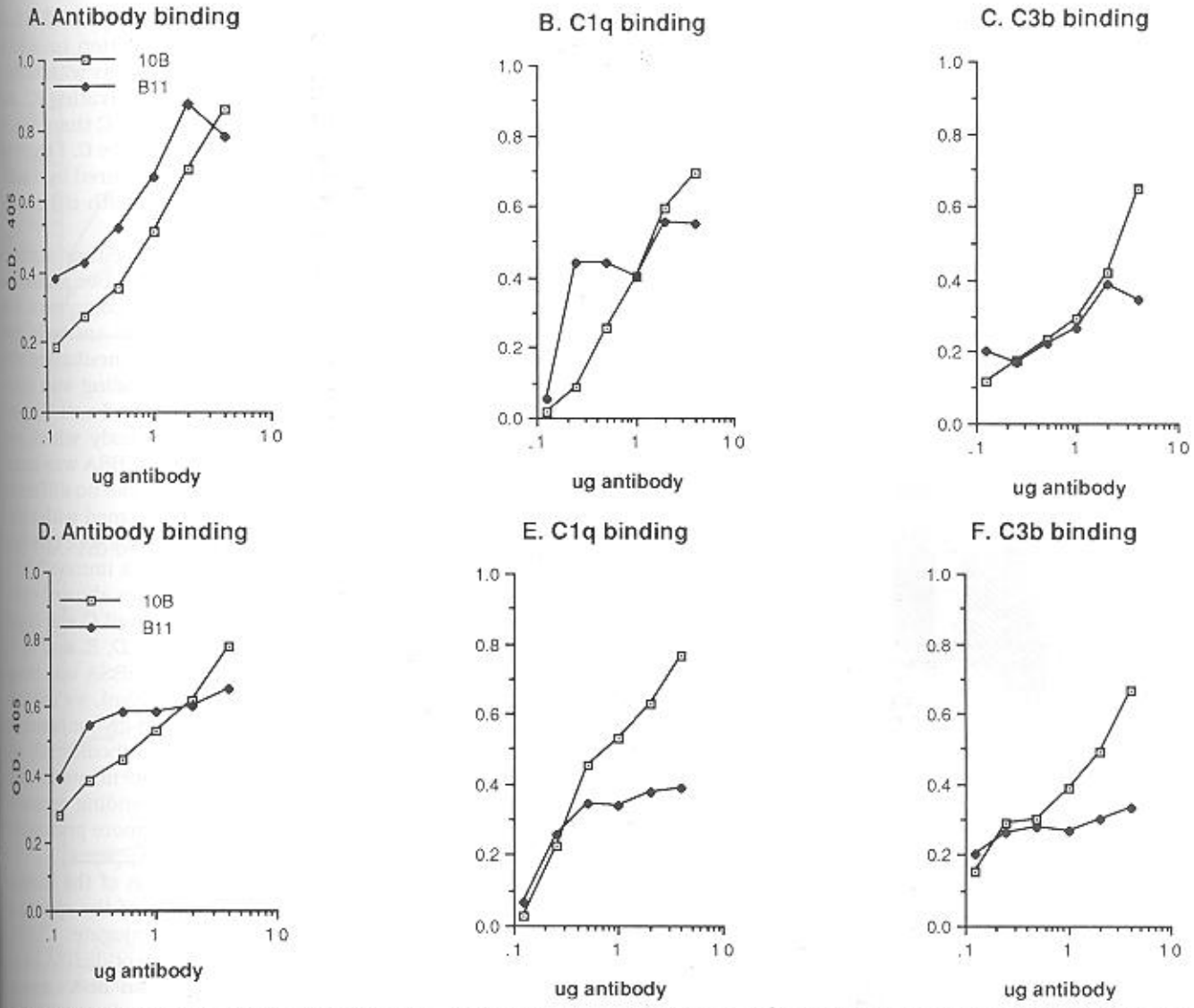


Figure 7. C activation by immobilized complexes. In *A*, *B*, and *C*, microtiter plates were coated with 5 μg/ml CYYYEEEEY:BSA (26 peptides per BSA) and antibody was added. The plates were then incubated with normal human serum (1/50 dilution). *A*, The amount of antibody bound to the plate was quantitated with anti-κ second antibody. *B*, C1q bound by the immobilized immune complex was quantitated with anti-C1q second antibody. C C3b bound by the IC was quantitated with anti-C3d second antibody. In *D*, *E*, and *F*, microtiter plates were coated with 1 μg/ml CYYYEEEEY:BSA (26/BSA). *D*, Antibody binding; *E*, C1q binding; *F*, C3b binding.

body (7S). Larger sizes could be a mixture of Ag with increasing numbers of antibodies, or complexes of Ag cross-linked by antibody. Figure 3 shows that the distribution of counts/min (^{125}I-Ag) in each size group was similar for the two antibodies. These data suggest that similar size complexes are formed in solution with either antibody. To examine the immune complex lattice, or Ag:antibody stiochiometry, immune complexes were formed in solution with antibody and ^{125}I-CYYYEEEEY:BSA and then precipitated with PEG. Both antibodies showed the same profiles of precipitation (Fig. 4) demonstrating that equal amounts of CYYYEEEEY:BSA were bound by equal quantities of antibody. Similar results were obtained with ammonium sulfate precipitation of the IC (data not shown). Ammonium sulfate precipitates complexes with high affinity antibodies, and usually lower affinity antibody complexes can be precipitated with PEG (11). We saw no differences in 10B and B11 immune complexes with either of these methods, suggesting that the complexes formed with either antibody are very much alike. Taken together, the results of the sucrose density gradients and the precipitation assays show that both antibodies form very similar soluble immune complexes with CYYYEEEEY:BSA.

When we measured binding of the antibodies to immobilized CYYYEEEEY:BSA, we found some differences between the antibodies. Antibody B11 bound the conjugate slightly better than antibody 10B at high density of immobilized Ag, and the binding of both antibodies decreased with a decrease in Ag density. However, the binding of antibody B11 decreased faster than that of 10B such that at low concentrations of peptide:BSA, antibody 10B bound as well or better than antibody B11 (data not shown). This led us to speculate that as the individual epitopes were spaced farther apart, antibody 10B would bind to conjugates that antibody B11 would not bind. To test this, CYYYEEEEY was conjugated to BSA at various peptide:BSA ratios and tested for antibody reactivity in an ELISA. The peptide:BSA ratios of the conjugates were estimated by the m.w. as determined by

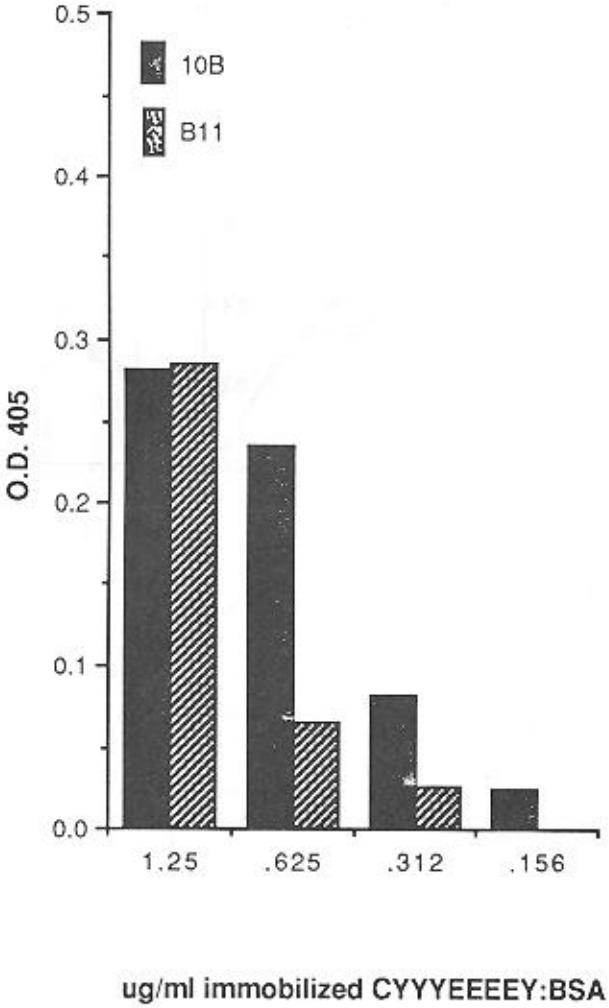


Figure 8. C1q binding by antibodies at decreasing concentrations of immobilized CYYYEEEEY:BSA. Microtiter plates were coated with CYYYEEEEY:BSA (26 peptides per BSA) at decreasing concentrations. Antibodies were reacted at 1 μg input. Antibody binding was equivalent for 10B and B11 at all Ag concentrations (data not shown).

SDS-PAGE. To preclude the event of high epitope density contributed by adjacent molecules, we chose the lowest Ag concentration (1 μg/ml, based on BSA content) that gave optimum binding of both antibodies. Figure 5 shows that as the peptide:BSA molar ratio of the conjugate was decreased, antibody 10B bound all conjugates, with slightly lower binding to the conjugate containing 5 peptides per molecule of BSA and lower still at 3 peptides per BSA. In contrast, the binding of antibody B11 decreased significantly at 5 peptides per BSA, and did not bind conjugates with only 3 peptides per BSA.

Both antibodies bound solution phase CYYYEEEEY:BSA equally, yet recognized the same Ag differently when it was immobilized. As the space between the epitopes was increased, either by the decreased concentration of immobilized Ag or by the decrease in the number of epitopes (peptides) per BSA, antibody 10B bound Ag with wider spaced epitopes than antibody B11 did. We next examined if the difference in C activation we had previously observed between these antibodies (8) would also depend on the spacing of epitopes.

C activation by immune complexed antibodies. We had previously shown that when equal amounts of 10B and B11 were bound to immobilized (Y,E)-A–K, antibody 10B activated C better and bound more C1q and C3b than antibody B11. Figure 6*A* shows C activation by soluble IC measured by the immune hemolysis assay with human serum for C. Both antibodies form C-activating IC, although antibody 10B was not better at activating C than B11. In fact, antibody B11 consumed slightly more C. Figure 6*B* shows C1q binding by soluble IC as measured by a solid-phase C1q binding ELISA. IC prepared with either antibody bound C1q equally.

Although the soluble complexes with either antibody activated C, immobilized complexes of the two antibodies differed. C activation by immobilized complexes was tested by incubating various amounts of antibody with immobilized CYYYEEEY:BSA and then incubating with normal human serum. C1q and C3b binding was measured with alkaline phosphatase-conjugated anti-C1q second antibody or anti-C3d second antibody which also recognizes C3b. When the CYYYEEEEY:BSA was immobilized at high density (5 μg/ml), there was no difference in the C1q binding by the complexes formed with either 10B or B11 (Fig. 7*B*), although 10B bound more C3b than B11 (Fig. 7*C*). When the peptide:BSA was immobilized at a lower density (1 μg/ml), both antibodies showed equivalent binding to the Ag, but 10B activated C and bound more C1q and C3b than B11 did (Fig. 7, *D*, *E*, and *F*). To look at C1q binding when CYYYEEEEY:BSA was immobilized at concentrations less than 1 μg/ml, we used the lowest antibody input (1 μg) that would give measurable C1q binding. At this antibody input, antibodies 10B and B11 bound equivalently at all Ag concentrations (data not shown). Figure 8 shows the C1q binding decreased with Ag density, but the decrease was more pronounced for antibody B11.

Finally, we examined the C activation of the immunized immune complexes as a function of the spacing of epitopes within the peptide:BSA conjugate. CYYYEEEY:BSA conjugates with varying peptide:BSA ratios were immobilized at 1 μg/ml (based on BSA content). Antibody 10B bound all conjugates with decreasing peptide:BSA ratios (Fig. 9*A*, see also Fig. 5*A*) and bound C1q and C3b at peptide:BSA ratios of 26–14. At 5 and 3 peptides:BSA, however, there was little to no C1q or C3b binding (Fig. 9, *B* and *C*). The binding of antibody B11 decreased with decreasing peptide:BSA ratios (Fig. 9*D*, see also Fig. 5*B*) and B11 bound C1q only at the 26 peptide:BSA ratio (Fig. 9*E*). C3b binding by B11 was measurable at 26–14 peptides:BSA, but was lower than the C3b binding by 10B (Fig. 9*F*).

DISCUSSION

Ig are globular proteins with different domains responsible for different functions. The variable regions recognize target Ag, whereas the constant regions control effector functions. In most cases mutations in the variable regions affect Ag binding characteristics (14) and mutations in the constant regions alter the effector functions (18–21). In some cases, however, mutations in the constant regions have resulted in altered Ag binding (22–24). Differences in C activation by IgG antibodies of the same subclass can often be attributed to differences in affinity or avidity (25–28) or in Ag:antibody stoichiometry (29–32). We previously described two IgG1 isomeric antibodies differing only in the V region, yet having altered C1q and C3b binding (8). We now extend the

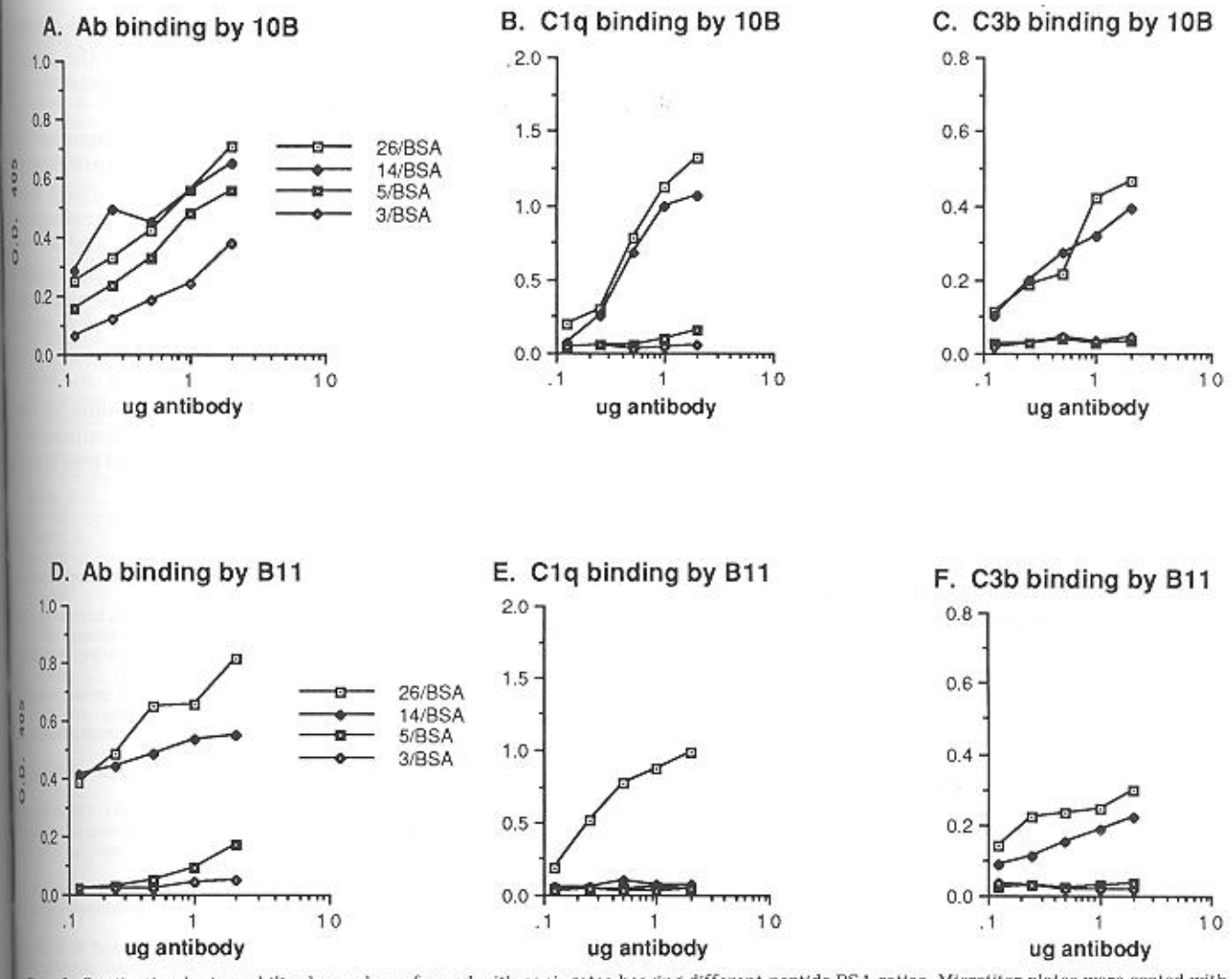


Figure 9. C activation by immobilized complexes formed with conjugates bearing different peptide:BSA ratios. Microtiter plates were coated with the different peptide:BSA conjugates at 1 μg/ml, based on BSA content and assayed for C1q and C3b binding after incubation with normal human serum. *A*, 10B antibody binding; *B*, C1q binding by 10B; *C*, C3b binding by 10B; *D*, B11 antibody binding; *E*, C1q binding by B11; *F*, C3b binding by B11.

early observations with a defined peptide as the epitope. We find that these antibodies have different capabilities to activate C when complexed to immobilized peptide:BSA conjugates, even in the context of equivalent antibody:Ag stoichiometry and epitope spacing.

In our earlier experiments, the binding characteristics of antibodies 10B and B11 to (Y,E)-A–K were examined. Although it appeared that 10B and B11 recognized different epitopes on (Y,E)-A–K, both antibodies bound similarly with the same relative affinity and strength of interaction. Also, both antibodies bound a linear polypeptide containing glutamic acid and tyrosine residues with the same association constant ($1.4–2.4 \times 10^{-7}$ M^{-1}). We wanted to standardize the epitope for the two antibodies and so decided to use a peptide:BSA conjugate as a defined, multivalent Ag in these assays. First, recognition sequences were identified by reacting the antibodies to a series of octapeptides consisting of Tyr and Glu, which were synthesized according to the protocol of Geysen et al. (33). We found that although antibody 10B recognized more sequences than B11 did, there were some peptides that were recognized by both antibodies. We chose one such peptide, CYYYEEEEY, to use in our study.

Both antibodies formed soluble complexes with CYYYEEEEY:BSA of similar size and Ag:antibody stoichiometry. The binding of CYYYEEEEY:BSA was equivalent for both antibodies as measured either by PEG precipitation of the complexes or ammonium sulfate precipitation. Because ammonium sulfate precipitation measures high affinity complexes and PEG precipitates low affinity complexes (11), the fact that these two assays gave the same results suggests there are not populations of high and low affinity antibodies in the mAb preparations. These soluble complexes activated C similarly, with complexes containing antibody B11 consuming slightly more C than antibody 10B. In contrast, immobilized complexes of the two antibodies exhibited significant differences. When immune complexes were formed in situ with either antibody and immobilized CYYYEEEEY:BSA, antibody binding and C activation was a function of epitope density. Increasing the space between epitopes, either by decreasing the concentration of the conjugate on the plate or by decreasing the number of peptides per BSA molecule, resulted in fewer antibodies binding to the immobilized Ag and lower C1q and C3b binding. Nevertheless, at equivalent antibody levels in the immobilized complexes, the decrease in C1q and C3b binding by antibody B11 was evident at higher epitope density than that by

10B. Thus, antibody 10B was able to activate C on immobilized Ag with wider spaced epitopes than antibody B11.

Antibody B11 consistently bound to immobilized CYYYEEEEY:BSA better than antibody 10B except at low peptide:BSA ratio (Figs 2*D*, 5, 7, *A* and *D*, and 9 *A* and *D*). Even so, B11 bound less C1q and C3b than 10B. We do not believe that is is due to monovalent binding vs bivalent binding of the different antibodies. It is unlikely that B11 binds monovalently, because it did not bind to CYYYEEEEY:BSA at low peptide:BSA ratios. If 10B can bind monovalently (and presumably bivalently as well) one would expect equivalent or higher detection of 10B in the antibody binding ELISA because for the same number of epitopes more antibodies would be bound at saturation. This was not seen. Also, monomerically bound antibody can activate C if appropriately aggregated, but is less effective than bivalently bound antibody (25). Taken with the solution data which show no difference in affinity, we believe both 10B and B11 bind bivalently to the immobilized CYYYEEEEY:BSA.

C activation by IgG requires the appropriate spacing of two Fc domains (34). In this system of immobilized CYYYEEEEY:BSA, we hypothesized that antibodies recognized the same surface of epitopes, yet 10B could achieve the appropriate Fc spacing for C1q binding whereas B11 could not. Our results could suggest that antibody 10B has greater flexibility than B11 and may achieve bivalent binding at lower epitope density than B11 can. Because both antibodies are IgG1, the difference in flexibility of the F(ab) arms must be a result of variable region differences in the H chain. To examine if mutations had arose in the the constant region genes which could account for these differences, we prepared mRNA from hybridomas expressing each antibody and performed cDNA sequencing. We found no differences in the constant regions between the two antibodies (data not shown).

An alternate explanation for the difference in C activation by the immobilized immune complexes may be that structural constraints are imposed on the antibodies when bound to immobilized Ag, and that this may alter their abilities to bind C1q. The C1q-binding site of IgG is in the CH_2 domain, and residues Glu-318, Lys-320, and Lys-322 have been shown to be necessary (6). More recently, Tao et al. (7) suggested that residue Tyr-296 on the opposite H chain may aso be involved. If antibodies 10B and B11 have different tortional strains put upon them when bound to low density, immobilized epitopes, then possibly the relationship of Tyr-296 to the opposite Glu-318-Lys-320-Lys-322 motif may be slightly altered in B11, preventing efficient binding of C1q.

Whether the difference between these antibodies is a function of flexibility of the F(ab) arms or a structural conformation, clearly, variable region differences can influence the activation of C by immobilized immune complexes. Notably, there are significant differences in C activation between soluble immune complexes and immobilized immune complexes prepared with the same Ag:antibody ratios. This finding has implications in the context of autoimmune and other inflammatory diseases where often tissue membrane components or Ag deposited therein are attacked by specific antibodies and C mediated lesions ensue. By extrapolating our results with these chimeric antibodies to the actions of autoan ies, we suggest that the variable region of the ant may direct its pathogenic potential independently recognition.

There is considerable effort directed at the ident tion of pathogenic subpopulations of autoantibodie 36) and the elucidation of variable gene usage a anti-DNA antibodies (37–42). It will be interesting if pathogenic antibodies can be experimentally dele diminished from the antibody repertoire in mod autoimmune disease by targeting those with particu gene usage, and whether this would result in a dec in pathology.

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REFERENCES

1. **Koffler, D.** 1974. Immunopathogenesis of systemic lupus er tosus. *Annu. Rev. Med. 25:149.*
2. **Koffler, D., G. Biesecker, and S. M. Katz.** 1982. Immuno nesis of systemic lupus erythematosus. *Arthritis Rheum. 2*
3. **Wilson, C. B., and F. J. Dixon.** 1974. Immunopathology and ulonephritis. *Annu. Rev. Med. 25:83.*
4. **Couser, W. G., and D. J. Salant.** 1980. In situ immune formation and glomerular injury. *Kidney Int. 17:1.*
5. **Schur, P. H.** 1982. Complement and lupus erythematosus. *Rheum. 25:793.*
6. **Duncan, A. R., and G. Winter.** 1988. The binding site for C *Nature 332:738.*
7. **Tao, M.-H., S. M. Canfield, and S. L. Morrison.** 1991. The dif ability of human IgG1 and IgG4 to active complement is det by the COOH-terminal sequence of the C_H2 domain. *J. Ex 173:1025.*
8. **Horgan, C., K. Brown, and S. H. Pincus.** 1990. Alteration chain V region affects complement activation by chimeric an *J. Immunol. 145:2527.*
9. **Pincus, S. H., S.-T. Ju, M. E. Dorf, L. P. Ewing, and B. A.** 1985. Variable regions of antibodies to synthetic polypep Characterization of an idiotope expressed on antibodies a factors. *Mol. Immunol. 22:455.*
10. **Carmack, C. E., and S. H. Pincus.** 1986. Variable regions of ies to synthetic polypeptides. II. Analysis of variable reg encoding antibodies specific for (T,G)A–L. *J. Immunol. 137*
11. **McGrath, H., Jr., and J. J. Biundo Jr.** 1985. A longitudinal high and low avidity antibodies to double-stranded DNA in lupus erythematosus. *Arthritis Rheum. 28:425.*
12. **Johns, P., and D. R. Stanworth.** 1976. A simple numerical for the construction of isokinetic sucrose density gradients application to the characterisation of immunoglobulin com *Immunol. Methods 10:231.*
13. **Horgan, C., and R. P. Taylor.** 1984. Quantitative analyse plement fixation in three immune complex systems. *Im 52:753.*
14. **Panka, D. J., M. Mudgett-Hunter, D. R. Parks, L. L. Peters Herzenberg, E. Haber, and M. N. Margolies.** 1988. Variab framework differences result in decreased or increased a variant anti-digoxin antibodies. *Proc. Natl. Acad. Sci. USA*
15. **Wallick, S. C., E. A. Kabat, and S. L. Morrison.** 1988. Glyc of a V_H residue of a monoclonal antibody against alpha(1–6 increases its affinity for antigen. *J. Exp. Med. 168:1099.*
16. **Wallick, S. C., E. A. Kabat, and S. L. Morrison.** 1989. The isotype and the J_K region on antigen binding and idiotype e by antibodies binding alpha(1–6) dextran. *J. Immunol. 142*
17. **Sharon, J., M. L. Gefter, L. J. Wysocki, and M. N. Margol** Recurrent somatic mutations in mouse antibodies to *p*-azo sonate increase affinity for hapten. *J. Immunol. 142:596.*
18. **Klein, M., N. Haeffner-Cavaillon, D. E. Isenman, C. Riv Navia, D. R. Davies, and K. J. Dorrington.** 1981. Expr biological effector functions by immunoglobulin G molecule the hinge region. *Proc. Natl. Acad. Sci. USA 78:524.*
19. **Nose, M., and T. Leanderson.** 1989. Substitution of aspar with aspartic acid in the Fc portion of mouse antibodies red capacity for Clq binding. *Eur. J. Immunol. 19:2179.*
20. **Nose, M., T. Okuda, M. Gidlund, U. Ramstedt, N. Okada, B. Heyman, M. Kyogoku, and H. Wigzell.** 1988. Mutant m antibodies with select alteration in complement activati Impact on immune complex functions in vivo. *J. Immunol.*

11. **Tao, M.-H., and S. L. Morrison.** 1989. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol. 143:2595.*
12. **Pollock, R. R., D. L. French, M. L. Gefter, and M. D. Scharff.** 1988. Identification of mutant monoclonal antibodies with increased antigen binding. *Proc. Natl. Acad. Sci. USA 85:2298.*
13. **French, D. L., R. R. Pollock, H. L. Aguila, and M. D. Scharff.** 1991. The molecular and biochemical characterization of mutant monoclonal antibodies with increased antigen binding. *J. Immunol. 146:2010.*
14. **Martel, F., R. Bazin, S. Verrette, and R. Lemieux.** 1988. Characterization of higher avidity monoclonal antibodies produced by murine B-cell hybridoma variants selected for increased antigen binding of membrane Ig. *J. Immunol. 141:1624.*
15. **Wong, J. T., and R. B. Colvin.** 1987. Bi-specific monoclonal antibodies: selective binding and complement fixation to cells that express two different surface antigens. *J. Immunol. 139:1369.*
16. **Fauci, A. S., M. M. Frank, and J. S. Johnson.** 1970. The relationship between antibody affinity and the efficiency of complement fixation. *J. Immunol. 105:215.*
17. **Ahlstedt, S., J. Holmgren, and L. A. Hanson.** 1974. Protective capacity of antibodies against *E. coli* O antigen with special reference to the avidity. *Int. Arch. Allergy Appl. Immunol. 46:470.*
18. **Blank, S. E., G. A. Leslie, and L. W. Clem.** 1972. Antibody affinity and valence in viral neutralization. *J. Immunol. 108:665.*
19. **Garred, P., T. E. Michaelsen, and A. Aase.** 1989. The IgG subclass pattern of complement activation depends on epitope density and antibody and complement concentration. *Scand. J. Immunol. 30:379.*
20. **Horgan, C., and W. Emlen.** 1987. Complement fixation by small, DNase-resistant DNA–anti-DNA immune complexes. *Mol. Immunol. 24:109.*
21. **Doekes, G., L. A. Van Es, and M. R. Daha.** 1984. Binding and activation of the first complement component by soluble immune complexes: effect of complex size and composition. *Scand. J. Immunol. 19:99.*
32. **Borsos, T., and A. Circolo.** 1983. Binding and activation of C1 by cell bound IgG: activation depends on cell surface hapten density. *Mol. Immunol. 20:433.*
33. **Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. G. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods 102:259.*
34. **Circolo, A., and T. Borsos.** 1982. Lysis of hapten-labeled cells by anti-hapten IgG and complement: effect of cell surface hapten density. *J. Immunol. 128:1118.*
35. **Hahn, B. H.** 1982. Characteristics of pathogenic subpopulations of antibodies to DNA. *Arthritis Rheum. 25:747.*
36. **Shoenfeld, Y., and E. Mozes.** 1990. Pathogenic idiotypes of autoantibodies in autoimmunity: lessons from new experimental models of SLE. *FASEB J. 4:2646.*
37. **Panosian-Sahakian, N., J. L. Klotz, F. Ebling, M. Kronenberg, and B. Hahn.** 1989. Diversity of Ig V gene segments found in anti-DNA autoantibodies from a single (NZB × NZW)F_1 mouse. *J. Immunol. 142:4500.*
38. **Hahn, B. H., and F. M. Ebling.** 1984. A public idiotypic determinant is present on spontaneous cationic IgG antibodies to DNA from mice of unrelated lupus-prone strains. *J. Immunol. 133:3015.*
39. **Livneh, A., A. Halpern, D. Perkins, A. Lazo, R. Halpern, and B. Diamond.** 1987. A monoclonal antibody to a cross-reactive idiotype on cationic human anti-DNA antibodies expressing lambda light chains: a new reagent to identify a potentially differential pathogenic subset. *J. Immunol. 138:123.*
40. **Davidson, A., A. Smith, J. Katz, J.-L. Preud'Homme, A. Solomon, and B. Diamond.** 1989. A cross-reactive idiotype on anti-DNA antibodies defines a H chain determinant present almost exclusively on IgG antibodies. *J. Immunol. 143:174.*
41. **Isenberg, D., C. Dudeney, F. Wojnaruska, B. S. Bhogal, J. Rauch, A. Schattner, Y. Naparstek, and D. Duggan.** 1985. Detection of cross reactive antiDNA antibody idiotypes on tissue-bound immunoglobulins from skin biopsies of lupus patients. *J. Immunol. 135:261.*
42. **O'Keefe, T. L., S. Bandyopadhyay, S. K. Datta, and T. Imanishi-Kari.** 1990. V region sequences of an idiotypically connected family of pathogenic anti-DNA autoantibodies. *J. Immunol. 144:4275.*