Borrower: LNU

Lending String: *HMS,HMS,HMS

Patron: Hernandez, Angela

Journal Title: Human antibodies and hybridomas.

Volume: 3 **Issue:** 3
Month/Year: 19920701 **Pages:** 153-71

Article Author:

Article Title: C, Horgan,; Variable region differences affect antibody binding to immobilized but not soluble antigen.

Imprint: Stoneham, MA, USA ; Butterworths, c1990-

ILL Number: 37603230

Call #: QA

Location:

Ariel 137.30.164.18
Charge
Maxcost: $50IFM/$30 EFTS

Shipping Address:
Earl K. Long Library
University of New Orleans
Interlibrary Loan
New Orleans, LA 70148

Fax: 504-280-3173
Ariel: 137.30.164.18

Variable region differences affect antibody binding to immobilized but not soluble antigen

Carol Horgan, Kathy Brown, and Seth H. Pincus

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

We have examined the antigen binding characteristics of two chimeric IgG_1 antibodies that differ only in the heavy chain variable region. Antibodies 10B and B11 were expressed from two different anti-(Tyr,Glu)-Ala-Lys murine V_H genes joined to human IgG_1 constant region genes in a murine anti-(Tyr,Glu)-Ala-Lys heavy chain loss variant hybridoma. The binding characteristics of the antibodies to (Tyr,Glu)-Ala-Lys and to a peptide conjugate, CYYYEEEEY:BSA, were measured in solution and solid phase assays. The antibodies exhibited similar affinities and binding characteristics when assayed in solution assays. However, when we measured binding of antibodies to immobilized antigens, we found that antibody affinity depended on the epitope density in the immobilized immune complexes. The binding of antibody 10B and of B11 to immobilized (Tyr,Glu)-Ala-Lys and to CYYYEEEEY:BSA were similar at high antigen density, but antibody B11 bound less well at lower antigen density. Fab fragments of 10B bound to immobilized (Tyr,Glu)-Ala-Lys and CYYYEEEEY:BSA, but Fab fragments of B11 did not bind to (Tyr,Glu)-Ala-Lys and bound less well to CYYYEEEEY:BSA than 10B Fabs.

Keywords: chimeric antibodies; affinity; soluble immune complexes; immobilized immune complexes

Introduction

Binding of antibody to antigen is usually characterized by affinity measurements made from solution assays such as equilibrium dialysis or radioimmunoprecipitation assays.[1] Affinity of antibodies can also be measured in solid phase competitive inhibition binding assays. We have characterized the binding of two chimeric IgG_1 antibodies to the branched chain polypeptide (Tyr,Glu)-Ala-Lys (which we abbreviate (Y,E)-A-K) and to a defined peptide:carrier antigen, CYYYEEEEY:BSA. We found significant differences in the antigen binding characteristics depending on whether the measurements were made in solution or solid phase assays. When antibodies 10B and B11 were incubated on immobilized antigen at limiting antigen density, 10B was able to bind to wider spaced epitopes than B11, suggesting 10B bound monovalently with higher affinity than B11 or possessed greater flexibility than B11.

Address reprint requests to Dr. Carol Horgan, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, MT 59840, USA.
Received for publication 24 March 1992.

Methods and materials

Antibodies and antigens

Antibodies 10B and B11 were expressed from murine V_H genes joined to human IgG_1 C_H genes. The constructs were transfected into murine hybridoma cells expressing murine light chain.[2] The antibodies were directed against the glutamic acid (E) and tyrosine (Y) residues of the synthetic polypeptide (Y,E)-A-K (ICN Biomedicals, Costa Mesa, CA, USA).[3] The antibodies differ only in the V_H region and have the same human C_H region and identical murine kappa light chains. The V_H region differences are shown in *Figure 1*.[4] Peptide CYYYEEEEY was synthesized by Dr. John Coligan of the NIH Biological Resources Branch. Maliemated BSA was purchased from Pierce Laboratories (Rockford, IL, USA), and the peptide was conjugated to it via the N-terminal Cys. Unincorporated peptide was removed by desalting the conjugation mixture on a G-50 spin column (Boeringher Mannheim, Indianapolis, IN, USA). The peptide:BSA ratio was determined to

```
                                         CDR1                 CDR2
                                        ------          -----------------
     1         11        21             31       41          51        61
10B  LQQPGAELVKPGASVKLSCKASGYTF TSYWMH WVKQRPGRGLEWIG RIDPNSGGTKTNQKFKS KATLTVD
B11  -------------------------- -N--I- -------------- -------A--------N -----IN

                                         CDR3
                                   ----------------
     71        81        91        101          111
10B  KPSSTAYIQLSSLTSEDSAVYYCAR REVDG YFDV   WGAGTTVTVSS
B11  ---N---N-------D--------- GYDYS -TANDY --Q--S-----
```

Figure 1 Amino acid sequence of heavy chain variable region. The deduced amino acid sequences of the heavy chain variable regions of antibodies 10B and B11 were derived from the nucleotide sequences of the original V_H genes.[4] These genes were used to construct the chimeric heavy chain genes.[2] After these genetic manipulations, we confirmed the sequence and found no mutations. Dashes indicate identity. The sequences of the heavy chain constant regions and the entire light chains of the two antibodies do not differ.

be 26 peptides:BSA by the migration of the conjugate on an SDS-PAGE gel.

Affinity purification of chimeric antibodies

Cell culture supernatants were applied to a (Y,E)-A-K affinity column[5] and eluted with 0.1 M NH_4OH. We discovered that antibody B11 did not bind to the column and could not be purified with this procedure. We therefore used protein G–Sepharose chromatography to purify both antibodies from culture supernatants.[2] Fab fractions of the antibodies were prepared by digestion of the antibodies with immobilized papain (Pierce) according to the manufacturer's protocol. Antigen binding equivalents of Fab fragments were calculated from the OD_{280} of the Fab preparations and adjusted for a molecular weight of one-third that of intact IgG when compared to μg of intact IgG in the antigen binding ELISA.

Evaluation of antibody binding to antigen

1. Solution assays. Peptide alone and peptide:BSA were radio-iodinated with ^{125}I using Iodogen (Pierce). Antibody binding of unconjugated peptides or peptide:BSA conjugates was determined by incubating ^{125}I-antigen with antibody and then precipitating the complexes in 7.5% polyethylene glycol (PEG) 8000[6] or saturated ammonium sulfate (SAS; Baker Chemical Co., Phillipsburg, NJ, USA). The percent ^{125}I-antigen precipitated was determined after spinning the samples for 5 minutes at 8,000 g at 4°C.

2. Solid phase assays. ELISAs were employed to measure the binding of antibody to immobilized antigen. Microtiter plates (Immunion 2; Dynatech Laboratories, Chantilly, VA, USA) were coated with (Y,E)-A–K, or CYYYEEEEY: BSA conjugates at various concentrations and then blocked with 1% BSA in PBS. Antibodies (at various concentrations) or Fab fragments were incubated on the immobilized antigen for 1 hour at 37°C and washed. Bound antibody was detected with alkaline phosphatase–conjugated anti-kappa antibody.[2] When irrelevant MAbs were assayed on the plates, there was no binding. Also, when maliemated BSA alone was used as immobilized antigen, there was no binding of antibodies 10B and B11. Measurements were always made in duplicate and the absorbance values were averaged. In all cases, there was less than 10% difference between duplicate measurements. In the standard ELISA, the plates were washed (five times) between all steps with PBS containing 0.05% Tween 20 in an automatic plate washer (model 1550; BioRad Laboratories, Richmond, CA, USA). To measure binding of Fab fragments we hand-washed the plates under a gentle stream of PBS (four times) instead of using the plate washer.

Results

Binding to (Y,E)-A–K–Sepharose

When cell supernatants were passed over a (Y,E),-A–K–Sepharose column, we found that antibody 10B bound to the column and could be eluted with 0.1 M NH_4OH. However, antibody B11 did not bind to the column. We originally interpreted this to mean that antibodies 10B and B11 had different affinities for (Y,E)-A–K. We subsequently used protein G–Sepharose to purify both antibodies for the binding sites.

Affinity measurements

We were unable to measure affinity constants for the two antibodies with (Y,E)-A–K using solution assays, since the (Y,E)-A–K precipitated even in the absence of antibody, but when a smaller, linear polypeptide (GT) was used, both antibodies were shown to have the same affinity for that antigen.[2] Using immobilized (Y,E)-A–K at high antigen density, the relative affinity and avidity of 10B and B11 were the same. We found no significant difference in on or off rates of antibody binding when binding to immobilized antigen was inhibited with free antigen.[2] Antibody 10B bound to the free peptide CYYYEEEEY in solution somewhat better than antibody B11 (K_a = 6.8 × 10^3 M^{-1}, and 4.5 × 10^3 M^{-1} for 10B and B11, respectively) as

measured by PEG precipitation *(Figure 2)*. However, when the peptide was conjugated to BSA to allow the formation of cross-linked immune complexes, both antibodies bound to CYYYEEEEY:BSA equally in solution as measured by PEG or SAS precipitation *(Figure 3)*. We have also measured the size of the soluble immune complexes by sucrose density gradient analysis,[7] and both antibodies formed complexes of similar sizes. Thus, both antibodies exhibited similar affinities for CYYYEEEEY:BSA and formed comparable immune complexes in solution.

Binding of immobilized antigen

We measured the binding to (Y,E)-A–K or CYYYEEEEY:BSA in solid phase assays. Microtiter plates were coated with (Y,E)-A–K or CYYYEEEEY:BSA at various concentrations and then blocked with BSA. Antibody 10B bound to immobilized (Y,E)-A–K equally well over a range of antigen densities, while antibody B11 bound less well at lower antigen density (*Figures 4A* and *4B*). Antibody B11 bound better to immobilized CYYYEEEEY:BSA than antibody 10B at high antigen densities, but as the antigen density decreased, antibody 10B was able to bind equally well or better than B11 (*Figures 4C* and *4D*).

Antibodies 10B and B11 bound soluble antigen equally well, yet they bound to immobilized antigen differently, depending on epitope density. This may indicate a requirement for bivalent binding in our ELISA, and if so, antibody 10B is able to bridge wider-spaced epitopes when binding to immobilized antigen. To test for monovalent binding we prepared Fab fragments and used them in the binding assay. Under our standard ELISA conditions, Fab fragments of 10B

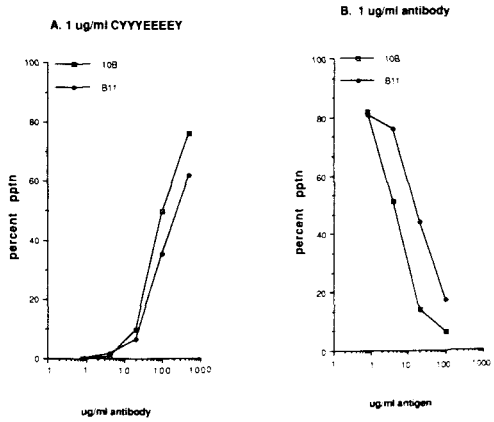

Figure 2 Binding CYYYEEEEY in solution. *(A)* Immune complexes were prepared with a constant amount of antigen, ^{125}I-CYYYEEEEY (1 μg/ml), with increasing concentrations of either antibody. 1% BSA was present as carrier. The mixtures were precipitated with an equal volume of 15% PEG. The percent of ^{125}I-CYYYEEEEY precipitated is shown on the *Y*-axis. *(B)* Immune complexes were prepared with a constant amount of antibody (10B or B11, 1 μg/ml), and increasing concentrations of ^{125}I-CYYYEEEEY. The mixtures were precipitated as in *(A)*.

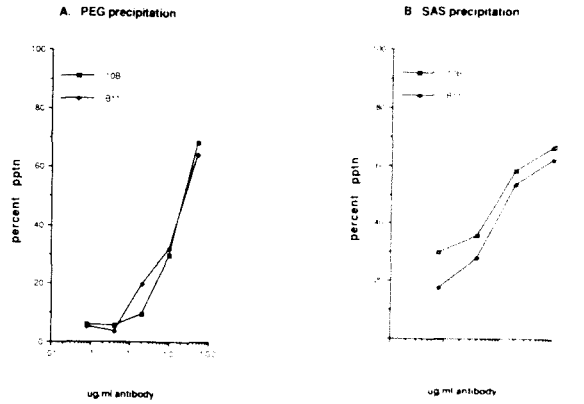

Figure 3 Binding to CYYYEEEEY BSA in solution. *(A)* Soluble immune complexes were formed with ^{125}I-CYYYEEEEY BSA at 1 μg/ml and increasing amounts of antibody. 1% BSA was present as carrier. The mixtures were precipitated in PEG, percent precipitation of ^{125}I-CYYYEEEEY:BSA is shown on the *Y*-axis. *(B)* The immune complexes were prepared as in *(A)* but precipitated with an equal volume of SAS.

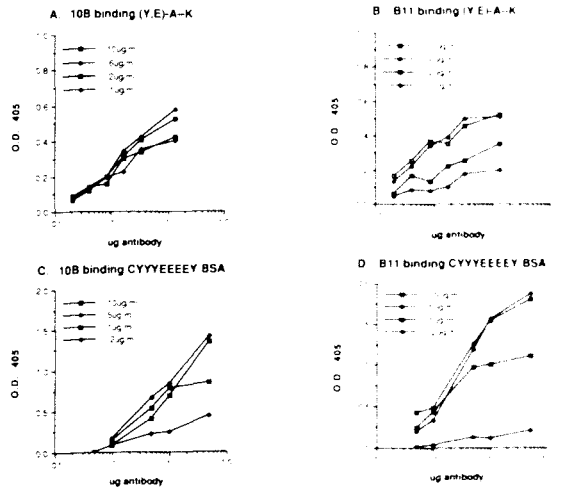

Figure 4 Binding of antibodies to immobilized (Y,E)-A–K and CYYYEEEEY:BSA conjugates. Microtiter plates were coated with (Y,E)-A–K or CYYYEEEEY:BSA at various concentrations and then blocked with BSA. Antibody binding to the immobilized antigen was detected with alkaline phosphatase–conjugated anti-kappa second antibody. *(A)* Binding of antibody 10B to (Y,E)-A–K. *(B)* Binding of antibody B11 to (Y,E)-A–K. *(C)* Binding of 10B to CYYYEEEEY:BSA. *(D)* Binding of B11 to CYYYEEEEY:BSA.

bound to CYYYEEEEY:BSA 20-fold lower than intact IgG for the same "antigen binding equivalents." Fab fragments of B11 did not bind in the standard assay *(Figure 5A)*. We altered the protocol to include a gentler washing step, and under our Fab ELISA conditions Fab 10B bound to immobilized (Y,E)-A–K and CYYYEEEEY:BSA, but Fab B11 did not bind to (Y,E)-A–K, and bound less well to CYYYEEEEY: BSA (*Figures 5B* and *C*).

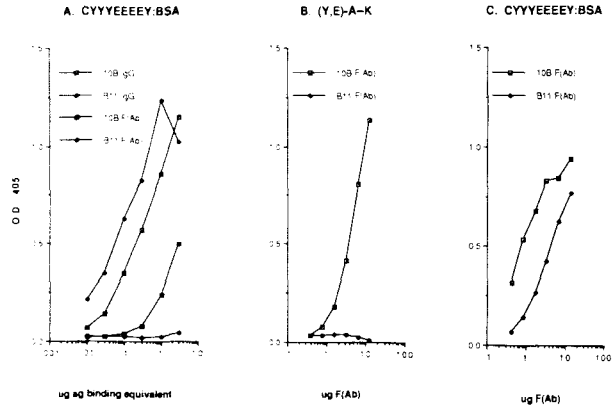

Figure 5 Binding of Fab fragments to immobilized antigens. *(A)* Microtiter plates were coated with CYYYEEEEY:BSA at 1 μg/ml, and the binding of intact IgG or Fab fragments was detected as described in *Figure 4*. *(B)* Binding of Fab fragments to immobilized (Y,E)-A-K at 8 μg/ml was measured in an adaptation of the ELISA protocol (Fab ELISA), which utilized gentler washing steps. *(C)* Binding of Fab fragments to CYYYEEEEY:BSA immobilized at 1 μg/ml was measured using the Fab ELISA protocol.

Discussion and conclusion

We previously described two IgG_1 chimeric antibodies differing only in the V region but having altered C1q and C3b binding.[2,7] We now present a more detailed examination of the antigen binding characteristics of these antibodies. We confirm and extend the observed differences between soluble and immobilized immune complexes prepared with these antibodies. Additionally we find that the apparent affinity of antibodies may differ under soluble versus solid phase conditions.

Our early observation that antibody 10B but not B11 could be purified on a (Y,E)-A-K–Sepharose column suggested that antibody 10B could bind bivalently to the column but antibody B11 could not. We suspected that the two antibodies recognized different epitopes on the (Y,E)-A-K molecule and that the epitopes recognized by 10B were closer together than those recognized by B11. Although 10B and B11 may have recognized different epitopes on (Y,E)-A-K, both antibodies bound to (Y,E)-A-K in an ELISA with the same relative affinity and strength of interaction. Also, both antibodies bound to a linear polypeptide containing glutamic acid and tyrosine residues with the same association constant ($1.4–2.4 \times 10^7\ M^{-1}$).[2] We wanted to standardize the epitope for the two antibodies and so decided to use a peptide:BSA conjugate as a defined, multivalent antigen in these assays.[7] To prepare an antigen with multiple copies of the epitope, we conjugated the peptide CYYYEEEEY to maleimated BSA via the NH_2-terminal C, which, in addition to providing the SH group for NH_2 terminal attachment acted as a spacer so that the sequence would be accessible to the antibodies.

Both antibodies formed soluble complexes with CYYYEEEEY:BSA of similar size and antigen:antibody stiochiometry. Their binding of CYYYEEEEY:BSA in solution was equivalent when measured by either PEG precipitation of the complexes or ammonium sulfate precipitation. Since ammonium sulfate precipitation measures high-affinity complexes and PEG precipitates low-affinity complexes,[6] the fact that these two assays gave the same results suggests that there are not populations of high- and low-affinity antibodies in the MAb preparations. In contrast to the soluble immune complexes, immobilized complexes of the two antibodies exhibited significant differences. When immune complexes were formed in situ with antibody and immobilized (Y,E)-A-K or CYYYEEEEY:BSA, antibody binding was a function of epitope density. Antibody 10B was able to bind to immobilized antigens at lower density than antibody B11. This suggested that antibody 10B could bridge wider-spaced epitopes than B11. We have also demonstrated this with CYYYEEEEY:BSA conjugates with decreasing peptide:BSA ratios.[7] If antibody 10B exhibits greater flexibility in binding to immobilized epitopes, then this is a function of the heavy chain variable domain, since the remainder of the molecule is identical for the two antibodies.[7]

We examined the binding of Fab fragments of the antibodies to immobilized antigen. Compared to intact IgG, the Fab fragments bound poorly (at least 20-fold lower per antigen binding equivalent). Under gentler conditions, where Fab binding was favored, Fab 10B bound to immobilized (Y,E)-A-K and CYYYEEEEY:BSA, but Fab B11 bound only to CYYYEEEEY:BSA. The binding of Fab B11 to CYYYEEEEY:BSA was lower than that of Fab 10B. This may be an artifact of the papain digestion process, since the binding of B11 (intact IgG) for immobilized CYYYEEEEY:BSA was consistently higher than that of 10B. At low density of antigen, our Fab ELISAs and IgG ELISAs would suggest that the affinity of antibody 10B is higher than that of antibody B11. However, at high antigen density, the differences in binding are not evident. Any differences in binding were noted only when measured on immobilized antigen. Affinity measurements of antibodies are commonly determined by solution assays. We suggest that the binding to immobilized antigen is different from binding to soluble antigen and may impose a structural constraint upon the IgG molecule that affects its relative affinity. We have suggested that such a constraint on the antibody also affects its ability to activate complement.[2,7]

Nomenclature

BSA	bovine serum albumin
C_H	heavy chain constant region
ELISA	enzyme-linked immunosorbent assay
GT	linear polypeptide of Tyr and Glu amino acids
MAb	monoclonal antibody
OD	optical density
PBS	phosphate-buffered saline
PEG	polyethylene glycol

SAS	saturated ammonium sulfate
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
V_H	heavy chain variable region
(Y,E)-A–K	branched chain polypeptide (Tyr,Glu)-Ala–Lys

Acknowledgment

We thank Susan Smaus for secretarial assistance. This work was funded in part by an Arthritis Investigator Award to Dr. Horgan.

References

1. Morel GA, Yarmush DM, Colton CK, Benjamin DC, Yarmush ML. Monoclonal antibodies to bovine serum albumin: affinity and specificity determinations. *Mol Immunol* 1988;25:7–15.
2. Horgan C, Brown K, Pincus SH. Alteration in heavy chain V region affects complement activation by chimeric antibodies. *J Immunol* 1990;145:2527–2532.
3. Pincus SH, Ju S-T, Dorf ME, Ewing LP, Araneo BA. Variable regions of antibodies to synthetic polypeptides. I. Characterization of an idiotope expressed on antibodies and T-cell factors. *Mol Immunol* 1985;22:455–461.
4. Carmack CE, Pincus SH. Variable regions of antibodies to synthetic polypeptides. II. Analysis of variable region genes encoding antibodies specific for (T,G)-A–L. *J Immunol* 1986;137:3983–3989.
5. Pincus SH, Sachs DH, Dickler HB. Production of antisera specific for idiotype(s) of murine anti-(T,G)-A–L antibodies. *J Immunol* 1978;121:1422–1427.
6. McGrath H Jr., Biundo JJ Jr. A longitudinal study of high and low avidity antibodies to double-stranded DNA in systemic lupus erythematosus. *Arthritis Rheum* 1985;28:425–430.
7. Horgan C, Brown K, Pincus, SH. Effect of heavy chain variable region on complement activation by immobilized immune complexes. 1991;149:127–135.