Fv Structure of Monoclonal Antibody II-481 Against Herpes Simplex Virus Fcγ-binding Glycoprotein gE Contains Immunodominant Complementarity Determining Region Epitopes that React with Human Immunoglobulin M Rheumatoid Factors

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Summary

Human immunoglobulin M (IgM) rheumatoid factors (RFs) show primary direct enzyme-linked immunosorbent assay (ELISA) reactivity with Fab rather than Fc fragments of monoclonal antibody (mAb) II-481 directed against the Fcγ-binding site of herpes simplex virus glycoprotein gE. This preferential anti-Fab specificity suggests that RFs react with antigen-binding portions of mAb II-481 as anti-idiotypic antibodies directed at the combining site regions of mAb reacting with the Fcγ-binding region of gE. Analysis of this idiotype–anti-idiotype reaction employed polymerase chain reaction amplification and sequencing of the variable heavy and light (VH and VL) regions of mAb II-481. When VH and VL regions of mAb II-481 were synthesized as overlapping 7-mer peptides on polypropylene pins, a panel of 10 polyclonal and 6 monoclonal human IgM RFs reacted primarily with epitopes within the three solvent-exposed mAb II-481 complementarity determining regions (CDRs). Preincubation of single CDR heptamer peptides with IgM RFs in free solution, resulted in 63–100% inhibition of RF binding to mAb II-481 on the ELISA plate, confirming the antigenic importance of linear CDR regions for RF reactivity. Combinations of two or three CDR peptides frequently produced 94–100% inhibition of RF binding to whole mAb II-481. Control peptides, singly or in combination, showed no inhibition. Computer modeling suggested that the RF-reactive mAb II-481 Fv region and a previously demonstrated RF-reactive CH3 epitope displayed considerable three-dimensional similarities in conformation. These studies may provide insight into limited shape homologies possibly involved in an RF anti-idiotypic reaction.

Human RFs represent one of the first types of autoantibodies ever described (1, 2), and over the five decades since their initial description, have provided a fascinating subject to study in parallel with the humoral immune response in rheumatoid arthritis (RA)[1] (3–7). The original observations on immunochemical and serologic reactions of RFs indicated that they represented autoantibodies directed at sites on the Fc region of human IgG. Indeed the primary anti-Fc specificity of human RFs was supported by formation of 22S and 11S complexes when 19S IgM or 7S IgG RFs reacted with autologous IgG (3–5). Moreover, anti-Fc specificity of human RFs was also supported by a multitude of studies of antiallotypic RF specificity for Gm determinants dependent on localized single or double amino acid substitutions within various regions of CH3 and CH2 of the Fc portion of IgG (8–10).

Recently, there have been a number of indications that, besides Fc, human RFs may have other parallel specificities such as the Fab portion of monoclonal mouse IgG antibodies directed at the Fcγ-binding region of the HSV glycoprotein E (gE) (11, 12). The reaction of human IgM RFs from patients with RA with the mouse monoclonal IgG-2b antibody II-481 to gE (the HSV-1 Fcγ-binding protein) was shown to resemble an idiotype–anti-idiotype reaction since, in most instances, RFs reacted much more strongly with Fab than with Fc fragments of mAb II-481 (12). If RFs from patients with RA react as anti-idiotypes against the Fv regions of mAb II-481 directed at the Fcγ-binding regions of gE, then the Fab portion and presumably the three CDRs of mAb

[1] *Abbreviations used in this paper:* FR1, framework region 1; gE, glycoprotein E; RA, rheumatoid arthritis.

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II-481 should contain immunodominant antigenic epitopes very similar or identical to RF-reactive regions on the Fc portion of IgG (13, 14). The present report provides evidence which supports that this is indeed the case. Moreover, data presented here also provide molecular and single amino acid epitope definition of an idiotype–anti-idiotype reaction between a common human autoantibody (RF) and the Fv portion of mAb II-481 directed at the Fcγ-binding region of the herpes simplex gE (14). The results presented here also suggest that similar conformations may be shared between the RF-reactive Fv regions of mAb II-481 and RF-reactive sites on CH3 of IgG.

Materials and Methods

Mouse Monoclonal Cell Line. The cell line II-481 B-2.4 producing the IgG-2b mouse mAb II-481 (15) directed at gE of HSV-1 was kindly provided by Dr. Pat Spear (Northwestern University Medical School, Chicago, IL) and was grown in OPTI-MEM media (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FCS, 0.3 mg glutamine, 100 U of penicillin, and 0.1 mg streptomycin (Sigma Chemical Co., St. Louis, MO) per ml. Cells were collected by centrifugation and supernatants tested for reactivity with isolated gE (5 μg/ml) as well as with whole HSV-1 by ELISA (11, 12). The cell line mAb product II-481 showed strong anti-gE specificity.

mRNA Isolation and cDNA Synthesis. mRNA was isolated from 3×10^8 cells using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). Briefly, cells were lysed in detergent-based buffer containing RNase and protein degrader and applied to oligo (dT) cellulose for adsorption. DNA, dissolved membranes, proteins, cell debris, and nonpolyadenylated RNAs were washed off the resin with salt buffers while the mRNA was eluted in the absence of salt. Purity and concentration of the mRNA was determined spectrophotometrically. Transcription of mRNA into first strand cDNA was performed according to the method of Gubler and Hoffman (16) with the cDNA Synthesis Kit from Boehringer Mannheim Biochemical (Chicago, IL). Briefly, the mRNA sample was heated to 65°C for 5 min and then incubated with AMV reverse transcriptase, oligo (dT) 15 primer or random primer, p(dN)6, RNase inhibitor, and deoxynucleotide mixture at 42°C for 1 h.

Oligonucleotide Primers. The oligonucleotide primers used in PCR are shown in Fig. 1. The 5′ primer for the light and 3′ primers for the L and H chains were those of Larrick and co-workers (17, 18) whereas the 5′ primer for the H chain was from Orlandi et al. (19). Originally, the primers were designed from known sequences of mouse IgG antibodies (20, 21). The 5′ primers were constructed from information available on conserved sequences of the first framework regions (FR1) of L and H chains, whereas the 3′ primers were designed for annealing within the constant regions of C κ L chains or CH^1 H chains. EcoRI and HindIII restriction sites were added to the 5′ and 3′ primers, respectively. Two bases (G) were added outside the restriction endonuclease site to improve enzyme digestion.

PCR. 2 μl of the cDNA-RNA hybrid and 100 pmol of the primer were added to the PCR mixture (GeneAmp; Perkin Elmer Cetus, Norwalk, CT) in a total volume of 100 μl. Amplification was done in a thermal cycler for 40 cycles (Perkin Elmer Cetus). Each cycle consisted of heating to 94°C for 1 min, primer annealing at 55°C for 3 min, and primer extension at 72°C for 3 min. PCR products were analyzed with ethidium bromide on a 2% agarose gel.

Cloning and DNA Sequencing. PCR bands were reamplified before being digested with EcoRI and HindIII restriction enzymes, isolated on gel, and ligated into plasmid pGem-3Zf(+) (Promega, Madison, WI). After selection of appropriate clones, each strand of the PCR product was sequenced by the dideoxynucleotide chain termination method (Sequenase version 2.0 kit; United States Biochemical Corp., Cleveland, OH) using T7 and Sp6 primers (Promega). mRNA from two separate cell clones was isolated, transformed to cDNA-RNA hybrids, and amplified by PCR to assure reproducibility of results. Two clones from each amplified VH and VL PCR product were selected and sequenced.

Study of Human RF Reactivity with VH and VL Primary Amino Acid Sequences of mAb II-481. The amino acid residues making up positions 20–105 on the VH and 14–100 on the VL regions of mAb II-481 were synthesized as overlapping 7 mers on Geysen pins as previously described (14, 22–27). Pins containing the VH or VL primary amino acid sequences were then tested directly with a panel of 10 affinity-isolated polyclonal and 6 monoclonal human IgM RFs obtained as previously described (22–24). The procedure for testing of the pins containing the overlapping 7 mers of VH or VL followed the procedure previously outlined in detail (14). Synthesis control pins containing the peptides with the sequences PLAC and GLAC were incubated with the positive control mAb (PT-02-20027; Cambridge Research Chemicals, Cambridge, MA). The next day, the pins were washed four times for 10 min with PBS containing 0.05% Tween-20. Depending on the primary reacting antibody, the pins were then incubated with peroxidase-conjugated goat $F(ab')_2$ anti-human IgM (for human IgM RF) or anti-mouse IgG (Jackson Laboratories, West Grove, PA) diluted in PBS containing 1% BSA, 200 μg/well, for 1.5 h at room temperature. After four wash steps, the pins were incubated with the O-phenylene-diamine substrate and color developed for 15 min. After completions of assays, a disruption procedure was employed to remove bound antibodies as previously described (14, 23, 24). Pins could then be reused 25–35 times before losing their antigenicity.

Synthesis of CDR Peptides and Inhibition of RF Binding to mAb II-481. After distinct RF-reactive linear sequences had been identified within the VL or VH CDRs using the pin ELISA assay, these reactive regions were synthesized as eight free heptamer peptides using the original Merrifield technique (28). These CDR peptides were used in competition/inhibition ELISA assays with RFs reacting with mAb II-481 on the ELISA plate. Titrations of increasing amounts of RF reacting with mAb II-481 (5 μg/ml) on the plate were used to establish the maximum binding concentration of each RF. Concentrations of RF producing 50% maximum binding were preincubated with a broad micromolar range of each of the eight CDR peptides before completion of the ELISA reaction between the RF and mAb II-481 on the plate. In many instances, various combinations of VL or VH mAb II-481 CDR peptides were studied to determine if inhibition of RF binding was increased using several peptides derived from different CDR regions. Several control peptides were used in parallel. A laminin peptide of the same length but no homologous sequence (CIKVSVS) was used as one control. Additional control peptides included: GMERVRWCATDGEG (melanoma peptide); LHNHYT (peptide from $C_\gamma 3$ of human IgG); CNSRQTDREDELI (Klebsiella peptide); ADAQTDREDLRTLLRY (HLA B27 peptide); AAEDWCKKGDT (IgA Cγ3 peptide); and NSRQTDR (Klebsiella peptide). Positive control inhibition was obtained with whole mAb II-481 or Fc of IgG.

Computer Analysis of II-481 Sequences. We used the previously established three-dimensional structure of the Fab portion of mouse IgG mAb D1.3 (29) available through the Brookhaven Data Base to provide a template for a tentative model of the VH and VL por-

tions of mAb II-481. Since the first eight amino acid residues of the VH and VL chains of mAb II-481 were missing because of primer annealing, these residues were replaced by the first eight amino acids from the VH chain of mAb Id B5.7 (30), and the missing residues for the VL chain by those from mAb A17 (31). In the comparable FRs of these latter mAbs, the amino acid residue homologies were 89% identical for the VH and 94% identical for VL to those of mAb II-481. Therefore, it was assumed that a similar homology existed for the first eight amino acids in the rather conserved FR1 regions of mAbs Id B5.7, A17, and II-481.

The sequences of the Fab portions of mouse mAb D1.3 and human mAb II-481 were aligned using the BESTFIT sub-routine of the GCG package (version 7.0; Genetics Computer Group, Madison, WI) (32). The overall identity was 80% for the L chain and 81% for the H chain and thus justified the use of standard methods of modeling by homology. The program used was Sybyl version 6.0 (Tripos Associates, Inc., St. Louis, MO) running on the VAX 4000-60 workstation. Structures were displayed on the Evans and Sutherland PS390 graphics system.

The loops of the H chain were modeled using the LOOP subroutine from Sybyl and the backbones displaying the smallest random mean square deviation values were selected. Individual amino acid substitutions were made using the MUTATE command in the BIOPOLYMER module. After the whole H chain was built, the structure was optimized for side-chain geometry and energy minimized employing the MAXIMIN2 module (1,000 cycles, no electrostatics, and vacuum conditions) with a standard Tripos force field.

To model the L chain of the Fab portion of IgG mAb II-481, the H chain of mAb D1.3 was replaced with the above constructed H chain of mAb II-481 and the resulting hybrid was used in the subsequent modeling. An attempt to model both chains separately did not provide satisfactory results. Therefore, the loops containing residues 26–32 and 90–96 of the L chain were modeled in the hybrid as described for the H chain. The whole structure was optimized for the side chain geometry and energy minimized as described above for the H chain modeling. In particular, the linear amino acid sequences within the three solvent-accessible CDR regions of the VL and VH of mAb II-481, which showed direct reactivity with RFs in pin ELISA, could be visualized and their physical proximity estimated. This strategy also allowed for construction of a tentative shape of the mAb II-481 combining site using space-filling spheres based on Connolly solvent-accessible surfaces (33). The whole modeling procedure was verified by using VL and VH sequences of other IgG 2bK mouse mAbs such as R19.9 (34, 35) which had already been extensively studied with respect to three-dimensional crystal structures. The validation process confirmed that the modeling steps we used accurately predicted most elements of Fv structure of previously studied mouse IgG 2bK antibodies where the crystal structures had been established. However, these predictions, of course, were not always perfect since the hypervariable regions of some antibodies actually assumed only one of several possible conformations.

Results

mRNA from the hybridoma cell line producing mAb II-481 was isolated and transformed by reverse transcriptase to cDNA-RNA hybrids. When the DNA sequence of the VL and VH regions of the Fv portion of mAb II-481 was amplified by PCR from the cDNA-RNA hybrids, the L chain primer pair (5′-EcoRI/FR1-ML [κ] and 3′-HindIII/ML [κ] constant) gave a major PCR product of ~400 bp whereas the H chain primer pair (5′-EcoRI/VH1 Back [FR1] and 3′-HindIII/MH [γ] constant) gave a band of ~410 bp (Fig. 2).

Mouse light kappa chain

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Amino acids 1-8
5': Eco RI/FR1-ML(kappa):
5'-GGGAATTCGA(CT)ATTGTG(AC)T(AG)AC(AC)CA(AG)(GT)(AC)TCAA-3'
     ------
      EcoRI


Amino acids 116-122
3': Hind III/ML(kappa) const:
5'-GGAAGCTTACTGGATGGTGGGAAGATGGA-3'
     ------
     HindIII
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Mouse heavy gamma chain

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Amino acid 1-8
5': Eco RI/VH1Back(FR1):
5'-GGGAATTCAGGT(CG)(AC)A(AG)CTGCAG(CG)AGTC(AT)GG-3'
     -----
     EcoRI


Amino acid 121-131
3': Hind III/MH(gamma)const:
5'-GGAAGCTTA(TC)CTCCACACACAGG(AG)(AG)CCAGTGGATAGAC-3'
     ------
     HindIII
```

Figure 1. 5′ and 3′ end oligonucleotide PCR primers for mouse variable light κ chain and mouse variable heavy γ chain. Bases in parentheses stands for equimolar amounts of bases at that position. The 5′ end restriction enzyme site EcoRI and the 3′ end restriction site HindIII are underlined.

After the PCR products were ligated into plasmid pGem 3Zf(+), they were cloned and sequenced. The nucleotide and amino acid sequences of the L and H chain variable regions including the four FR and three CDR regions are shown in Fig. 3, *A* and *B*. The variable L and H chain regions consisted of 107 and 113 amino acid residues, respectively. The L chain was identified as mouse κ subgroup I and the H chain as subgroup IB (21).

mRNA of two independent clones from the mAb II-481–producing cell line was isolated and from each PCR-amplified mRNA-cDNA hybrid, two clones were selected and completely sequenced. In all, four separate sequences from the VL and four from the VH were obtained. No mutations were detected in the four sequenced clones from L chain. In one of the four clones from the H chain, G was substituted for C at amino acid 77, changing the corresponding amino acid residue Gln to His at this position.

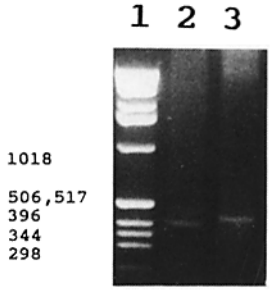


Figure 2. PCR products of the variable part of mouse mAb II-481 L (lane *2*) and H (lane *3*) chains. 10 μl from a 100-μl PCR reaction was applied in each lane on a 2% agarose gel stained with ethidium bromide together with a 1-kb marker ladder (lane *1*).

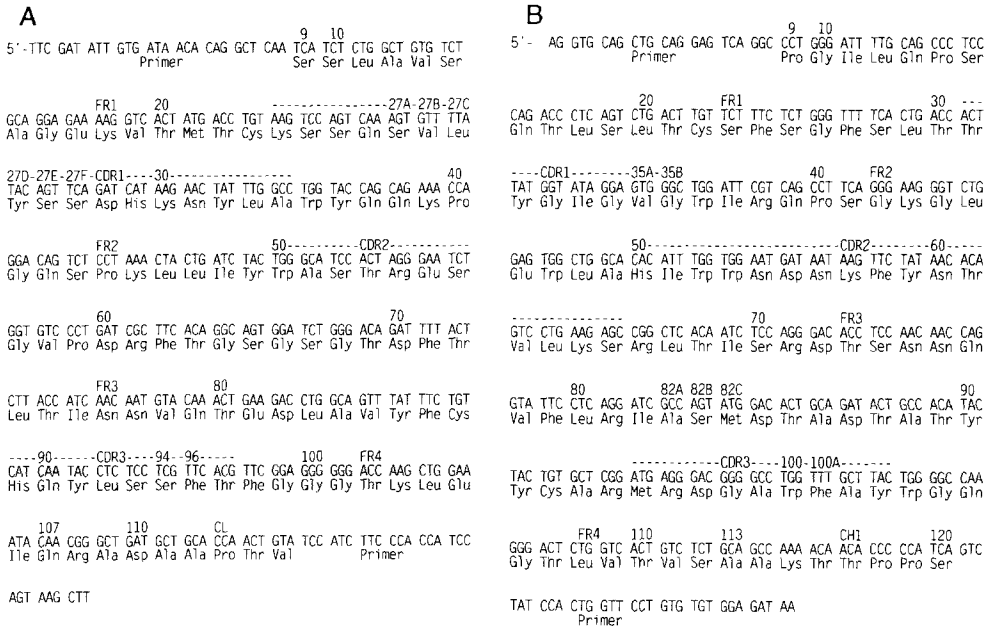


Figure 3. (*A*) Sequence of the variable part of mouse mAb II-481 L chain (amino acids 9–107) with FR and CDR. Primers and part of constant light (CL) chain sequences (amino acids 108–115 are also shown). (*B*) Sequence of the variable part of mouse mAb II-481 H chain (amino acids 1–113) with FR and CDR. Primers and part of constant H chain region 1 (CH1; amino acids 114–120) sequences are also shown. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X81462 (H chain) and X81463 (L chain).

Human Polyclonal and Monoclonal RFs React with 7 mers of mAb II-481 VH and VL Primary Amino Acid Sequence. When the entire VH sequence of mAb II-481 was synthesized as overlapping 7 mers on Geysen pins and tested for reactivity with polyclonal RFs, four to five major regions of positive reactivity were identified. Localized major positively reacting regions were found at 7 mers beginning with residues 20–27, 32–39, 44–58, and 94–105 on the VH segment. Some RFs also showed moderate reactions with 7 mers at positions 65–68. All of the major reactive VH-region sites were solvent accessible. Some variability was noted from RF to RF in terms of exact localization and numbers of reactive VH regions. However, the overall patterns of reactive ELISA profiles for most polyclonal RFs were remarkably similar. An example of results obtained with individual RFs is shown in Fig. 4 *A*.

When the VL sequence of mAb II-481 was also tested with the same panel of polyclonal RFs, localized reactive epitopes were found at 7 mers beginning with residues 25–32, 31–38,

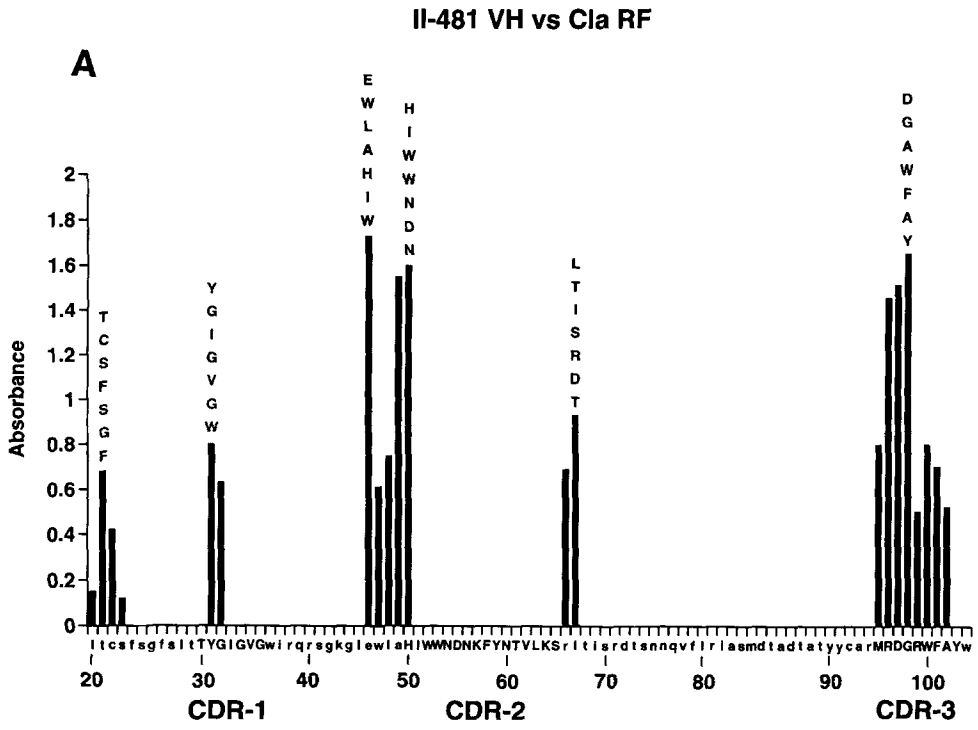
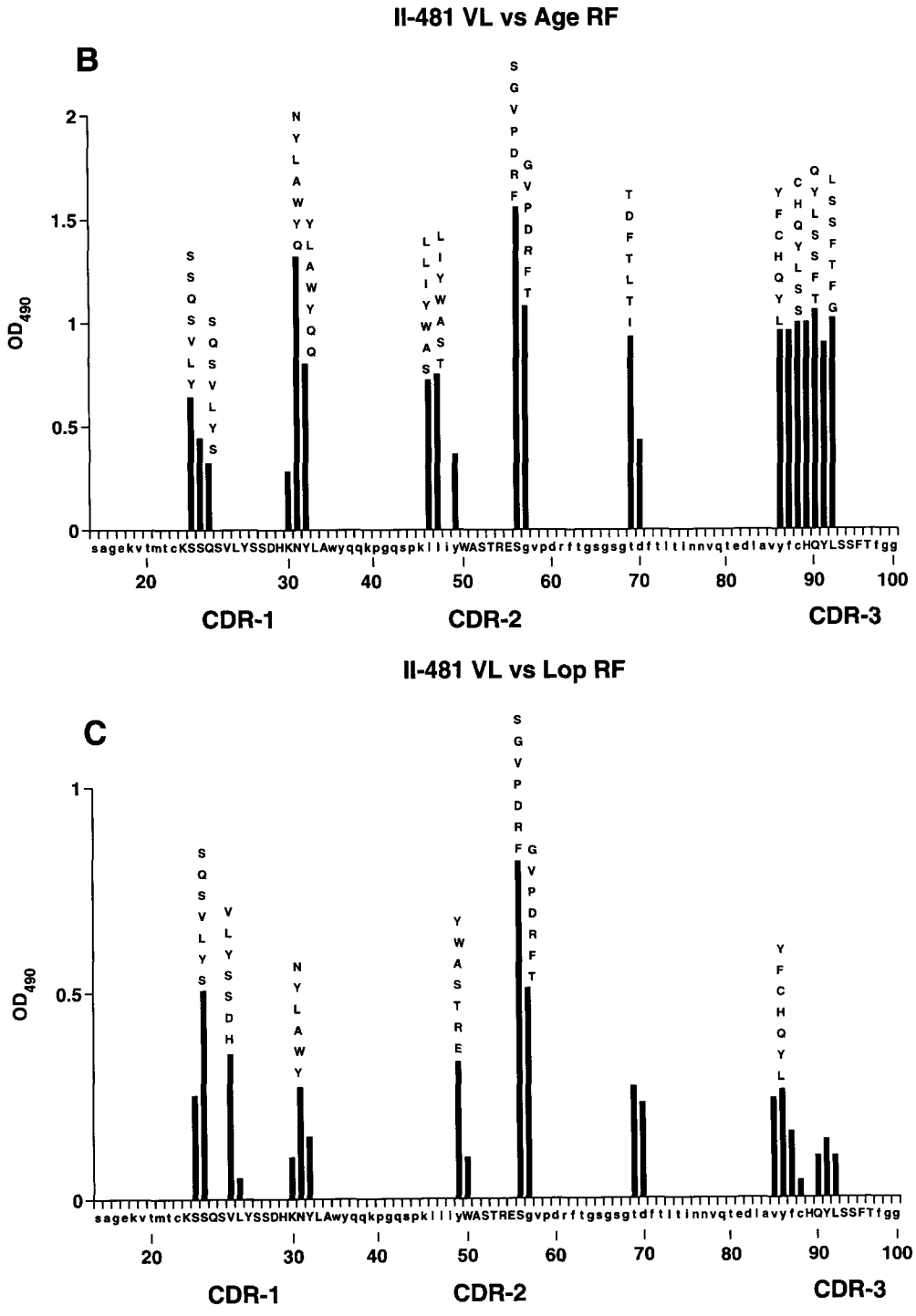


Figure 4. (*A*) Example of results of pin ELISA assays done with pins encompassing residues 20–105 of the variable H chain of mouse monoclonal II-481 as overlapping heptamers on each pin. The residue numbers are shown on the horizontal axis. Residues within the CDR-1, -2, and -3 regions are shown in capital letters. Optical densities of ELISA assays done with 1 of 10 polyclonal IgM RF (Cla) and the heptamer peptide on each pin are shown as vertical bars. (*B* and *C*) Examples of result of pin ELISA assay done with pins encompassing residues 14–100 of the variable L chain of mouse monoclonal II-481 as overlapping heptamers on each pin. The residue numbers are shown on horizontal axis and the residues within the three CDR regions are designated in capital letters. Optical densities of ELISA assays done with 2 of the 10 polyclonal IgM RFs and the heptamer peptide on each pin are shown as vertical bars.

46–53, 56–64, 69–72, and 85–97. All of these reactive regions were judged to be solvent accessible. Again, some variability was noted from RF to RF in terms of exact localization and number of the most reactive VL regions. Examples of the profiles of RF VL reactivity are shown in Fig. 4, *B* and *C*. No correlation could be found between patterns of RF CDR or VH/VL reactivity and RF anti-IgG subclass or Gm specificity.

Monoclonal human IgM RFs generated from B cells of patients with RA were also tested with the overlapping 7 mers of the VH and VL II-481 sequence. Major VH and VL regions of RF reactivity were similar to those previously observed with the polyclonal IgM RFs. Individual monoclonal RFs reacted with all or some of these sites. Examples of monoclonal RF patterns of VH and VL reactivity are shown in Fig. 5, *A* and *B*. Overall, the monoclonal RFs duplicated



similar patterns recorded with many of the polyclonal RFs previously studied, although exact localization and numbers of reactive regions varied slightly.

ELISAs performed with control antibodies including normal polyclonal human IgM without RF activity as well as monoclonal Waldenström's IgM paraproteins, also without RF activity, were completely negative with the entire set of synthetic peptides corresponding to the VH and VL of mAb II-481 (data not shown).

Human Polyclonal and Monoclonal IgM RFs React Primarily with CDR Regions on VH and VL Chains of mAb II-481. After the reactivity profile of each polyclonal and monoclonal human IgM RF against the VH and VL overlapping 7 mers of mAb II-481 was established by pin ELISA, it became clear that both polyclonal and monoclonal RFs reacted predominantly with regions on both VH and VL chains which were extremely close to or within CDR-1, -2, and -3 regions of mAb II-481. All of the reactive CDR regions were solvent accessible and therefore could be considered as important potential antigenic sites.

The profiles of reactivity of polyclonal IgM RF with respect to the VH CDR regions are shown in Table 1. It can be seen that 6 of the 10 polyclonal IgM RFs tested reacted with heptamers that included one or more amino acid residues from all three CDR regions. Four RFs reacted with two of the three CDRs. The profiles of the same 10 polyclonal RFs'

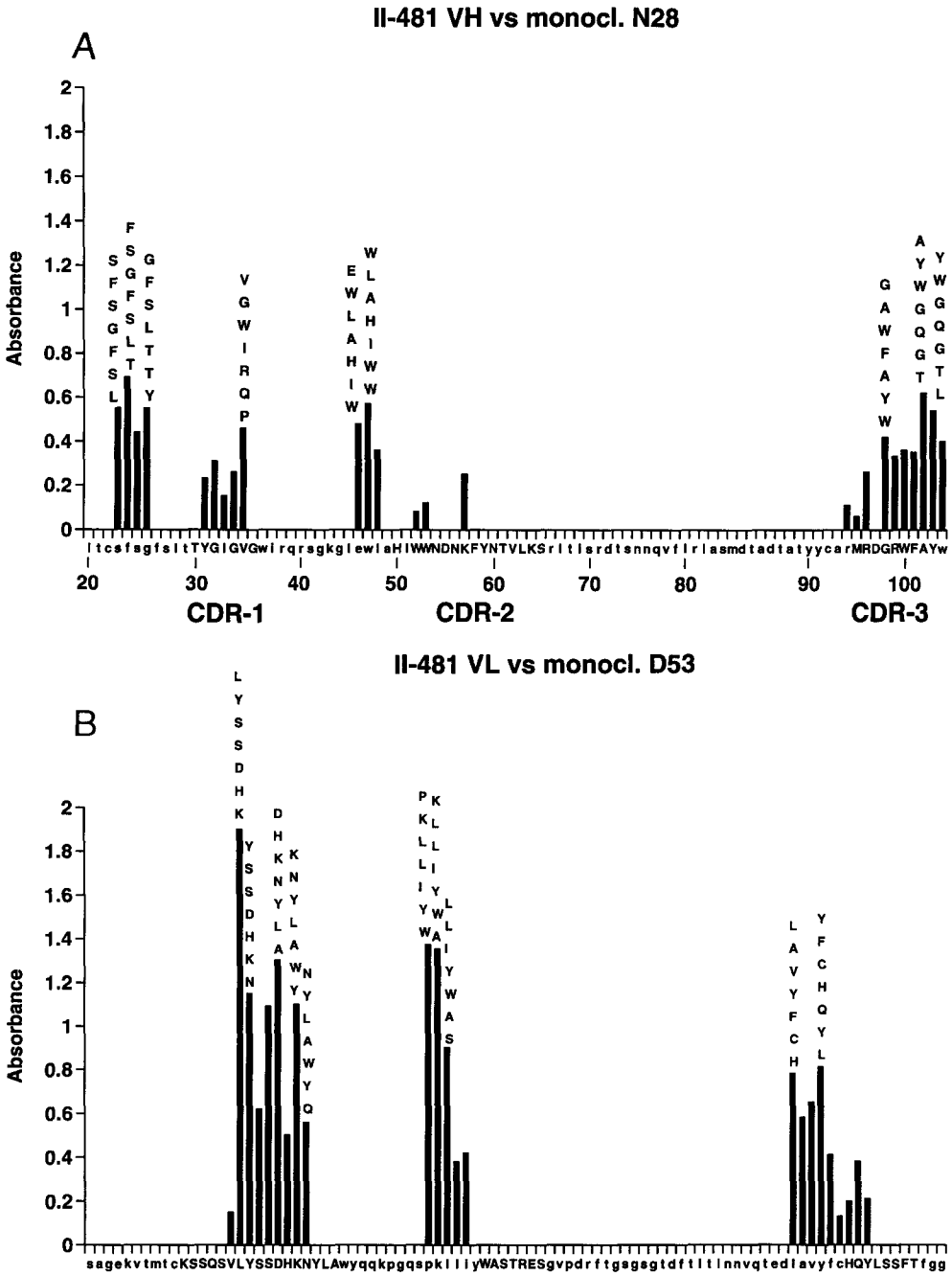


Figure 5. (*A*) Example of results of pin ELISA assays done with pins encompassing residues 20–105 of the variable H chain of mouse monoclonal II-481 as overlapping heptamers on each pin. The residue numbers beginning each 7 mer are shown on the horizontal axis. Optical densities of ELISA assays done with monoclonal IgM RF N28 and the heptamer peptide on each pin are shown as vertical bars. (*B*) Example of result of pin ELISA assay done with pins encompassing residues 14-100 of the variable L chain of mouse monoclonal II-481 as overlapping heptamers on each pin. Optical densities of ELISA assays done with one of the six monoclonal IgM RFs and the heptamer peptide on each pin are shown as vertical bars.

reactivity with mAb II-481 VL 7 mers are shown in Table 2. Five RFs reacted with epitopes from all three CDR regions, two reacted with two CDRs, and three with only one CDR. Of interest was the epitope represented by SGVPDRF which occurred largely just after the second CDR in the FR3 region. This region contained only one residue (the serine at position 56) within the CDR-2.

For monoclonal IgM RFs, the profile of VH reactivity is shown in Table 3. Four of the six monoclonal RFs reacted with 7 mers which included one or more residues from all three CDRs. One monoclonal RF reacted with two CDRs and one (H4) with only one CDR. The VL reactivity profile for three monoclonal IgM RF is shown in Table 4. Two of three showed reactions with residues within all three CDRs; the third (H4) showed a strong reaction with only one CDR.

Cross-inhibition of IgM RF Reacting with mAb II-481 Using mAb II-481 CDR Peptides. A series of inhibition experiments was conducted in which polyclonal IgM RFs known to react with the Fab portion of mAb II-481 were preincubated with peptides synthesized from the CDR RF-reactive regions of mAb II-481 before completion of the ELISA reaction between RFs and mAb II-481 on the ELISA plate.

Results of competition ELISA assays using peptides from the three CDRs of mAb II-481 to inhibit the binding of various polyclonal IgM RFs to II-481 on the ELISA plate are shown in Table 5 and Fig. 6. It can be seen that the patterns of CDR peptide inhibition were quite different for each RF tested and that within the profile of peptides tested, at least one CDR 7-mer peptide showed maximum inhibition for each RF (63–100%). Results of these inhibition experiments correlated quite well with the pin ELISA direct binding assays where the entire II-481 VH or VL linear sequence had been previously assayed. Of interest was the major degree of inhibition (62–90%) recorded with the SGVPDRF peptide from the CDR-1 VL region and the high levels of inhibition (80–100%) found with CDR-2 VH peptide HIWWNDN when individual polyclonal IgM RFs were tested. Fig. 6 shows final 96% inhibition for CDR-2 VH peptide HIWWNDN with RF AGER and a broad range of lesser inhibition with the other CDR peptides tested. No inhibition was recorded

Table 1. *Polyclonal IgM RF profiles with Variable Heavy Chain of Mouse mAb II-481*

Polyclonal IgM RF	CDR1 Region	CDR2 Region	CDR3 Region
Age	YG I GVGW*	EWLAH I W	DGAWFAY
			RDGAWFA
			MRDGAWF
Abr	YG I GVGW	LEWLAH I	RDGAWFA
		AH I WWND	DGAWFAY
Cla	YG I GVGW	EWLAH I W	DGAWFAY
		H I WWNDN	RDGAWFA
		(LT I SRDT)	MRDGAWF
Har	None	GLEWLAH	RDGAWFA
		H I WWNDN	MRDGAWF
Lop	YG I GVGW	GLEWLAH	DGAWFAY
		LEWLAH I	RDGAWFA
		H I WWNDN	
New	TYG I GVG	FYNTVLK	None
McC	TTYG I GV	GLEWLAH	MRDGAWF
		EWLAH I W	RDGAWFA
		H I WWNDN	FAYWGQG
Sor	G I GVGW I	EWLAH I W	AWFAYWG
		WLAH I WW	GAWFAYW
		LAH I WWN	DGAWFAY
Str	None	GLEWLAH	MRDGAWF
		H I WWNDN	
Wea	None	H I WWNDN	RDGAWFA

* Reactive residues within or adjacent to each CDR are shown as single letter amino acid designation; residues underlined are actually within the respective CDRs. When several reactive 7 mers are shown beneath a single CDR, the first sequence listed showed maximum reactivity with test RF. Residues shown in parentheses were weakly positive in ELISA reactions with RFs.

Table 2. *Polyclonal IgM RF Profiles with Variable L Chain of Mouse mAb II-481*

Polyclonal IgM RF	CDR1 Region	CDR2 Region	CDR3 Region
Age	SSQSVLY*	LLIYWAS	YFCHQYL
	SQSVLYS	LIYWAST	FCHQYLS
	NYLAWYQ	SGVPDRF	CHQYLSS
	YLAWYQQ	GVPDRFT	HQYLSSF
Abr	VLYSSDH	YWASTRE	(HQYLSFT)
		SGVPDRF	
		GVPDRFT	
Cla	VLYSSDH	LIYWAST	YFCHQYL
	NYLAWYQ	SGVPDRF	
		GVPDRFT	
Har	None	SGVPDRF	None
Lop	SQSVLYS	YWASTRE	YFCHQYL
	SSQSVLY	SGVPDRF	
	VLYSSDH	GVPDRFT	
	NYLAWY		
McC	NYLAWYQ	SGVPDRF	AVYFCHQ
	YLAWYQQ	GVPDRFT	
New	KVTMTCK	LIYWASTR	None
	VTMTCKS		
	TMTCKSS		
Sor	VLYSSDH	YWASTR	None
	NYLAWYQ	SGVPDRF	
		GVPDRFT	
Sti	None	SGVPDRF	None
		GVPDRFT	
Wea	None	SGVPDRF	None
		GVPDRFT	

* Residues underlined are actually within respective CDR. The first 7 mers listed showed maximum RF reactivity and those shown below, positive RF ELISA reactions but of lesser magnitude.

for the control laminin 7-mer peptide CIKVAVS or the five other control peptides, and complete inhibition was always recorded when either whole Fc fragment or mAb II-481 was used as inhibitor. When mAb II-481 CDR peptides were used as inhibitors of RFs binding to whole II-481 on the ELISA plate, a broad range of micromolar concentrations of peptides were tested and maximum inhibition of RF binding was found with highest peptide concentrations tested. By contrast, much lower concentrations of whole Fc or whole mAb II-481 were sufficient for complete inhibition (Fig. 6).

It can be seen from Table 5 that with all RFs tested for inhibition using single CDR peptides, usually one or two such peptides showed highest levels of inhibition varying from 28–100%. In no instance did any of the panel of six control peptides show inhibition. Accordingly, a second kind of inhibition experiment was conducted in which combinations of two or three II-481 CDR peptides were tested for inhibition of RF binding to mAb II-481 on the ELISA plate. Typical results are shown in Table 6. It can be seen that combinations of two or three CDR peptides almost always showed an increment of inhibition beyond what had been recorded with the single CDR peptides alone. In some instances, the percent inhibition was actually higher than the sum of inhibitions with the single peptides tested alone as inhibitors, and in only two instances (RF LOP and ABR), the two inhibiting peptides studied together did not far surpass the degree of inhibition recorded when the CDR peptides were tested alone. From Table 6 it can also be seen that combinations of two or three CDR peptides produced 50–100% inhibition with 88–100% inhibition in 10 of 12 experiments. The fact that two or sometimes three CDR peptides produced an increment in observed inhibition of RF binding over what had been recorded with single CDR peptides provided additional confirmation of the validity of the assays.

Table 3. *Monoclonal IgM RF Profiles with Variable H Chain of Mouse mAb II-481*

Monoclonal IgM RF	CDR1 Region	CDR2 Region	CDR3 Region
D53	YGIGVGW*	EWLAHIW	DGAWFAY
	GIGVGWI	WWNDNKF	GAWFAYW
	IGVGWIR	WNDNKFY	AWFAYWG
N28	SFSGFSL	EWLAHIW	GAWFAYW
	FSGFSLT	WLAHIWW	AYWGQGT
	GFSLTTY	LAHIWWN	
	GIGVGWI		
	GVGWIRQ		
	VGWIRQP		
H6	GFSLTTY	EWLAHIW	MRDGAWF
	SLTTYGI	WLAHIWW	RDGAWFA
	YGIGVGW	LAHIWWN	DGAWFAY
	GIGVGWI		GAWFAYW
			AWFAYWG
			WFAYWGQ
H4	None	EWLAHIW	None
		WWNDNKF	
		WNDNKFY	
		FYNTVLK	
RF 114	VGWIRQP	EWLAHIW	None
		WLAHIWW	
G4	TYGIGVG	EWLAHIW	YYCARMR
	GVGWIRQ	WLAHIWW	
		KFYNTVL	

* Residues within respective CDR are shown as underlined. When several reactive 7 mers are shown beneath a single CDR, the first sequence listed showed maximum RF reactivity.

Table 4. *Monoclonal IgM RF Profiles with Variable L Chain of Mouse mAb II-481*

Monoclonal IgM RF	CDR1 Region	CDR2 Region	CDR3 Region
D53	LYSSDHK*	PKLLIYW	YFCHQYL
	YSSDHKN	KLLIYWA	LAVYFCH
	SDHKNYL	LLIYWAS	VYFCHQY
	DHKNYLA		
	KNYLAWY		
N28	SSOSVLY	LIYWAST	QYLSSFT
	SOSVLYS		
	KNYLAWY		
	NYLAWYQ		
H4	LYSSDHK	(LIYWAST)‡	None
	YSSDHKN		

* Residues within respective CDR are shown as underlined.
‡ H4 reaction with LIYWAST was borderline in optical density (OD 0.250).

Table 5. *Inhibition of Polyclonal IgM RF Binding to mAb II-481 Using 7-mer Peptides from the VL and VH CDRs of mAb II-481*

mAb II-481 CDR	Actual sequence of CDR peptide	Polyclonal IgM RF tested							
		CLAU	AGER	LOP	STR	WEAK	MCC	SOR	ABR
CDR-1 VL	NYLAWYQ	40*	45	20	28	30	40	45	12
CDR-1 VL	SQSVLYS	10	12	18	0	0	0	0	6
CDR-2 VL	SGVPDRF	15	28	25	18	[72‡]	[80]	[90]	[62]
CDR-2 VL	LIYWAST	20	32	8	8	5	8	14	4
CDR-3 VL	YFCHQYL	10	44	10	0	0	14	0	0
CDR-1 VH	YGIGVGW	[100]	24	12	0	0	14	40	12
CDR-2 VH	HIWWNDN	32	[96]	[88]	10	26	16	8	[80]
CDR-3 VH	RDGAWFA	0	40	45	[63]	4	32	14	22
Control peptides:									
Laminin	CIKVAVS	0	0	0	0	0	0	0	0
Human $C_\gamma 3$	LHNHYT	0	0	0	0	0	0	0	0
Klebsiella	CNSRQTDREDELI	0	0	0	0	0	0	0	0
HLA B27	AKAQTDREDLRTLLRY	0	0	0	0	0	0	0	0
IgA $C_\gamma 3$	AAEDWCKKGDT	0	0	0	0	0	0	0	0
Klebsiella	NSRQTDR	0	0	0	0	0	0	0	0
Whole Fc human IgG		100	100	100	100	100	100	100	100
mAb II-481		100	100	100	100	100	100	100	100

* Numbers refer to percent inhibition of each RF binding to mAb II-481 using preincubation with the CDR peptide tested.
‡ Experiments where a single CDR peptide produced 60% or more inhibition are boxed.

Also, in no instance did any combination of two or three control peptides show any inhibition of RF binding (data not shown).

The finding that the isolated immunoreactive peptides such as YGIGVGW from the VH CDR-1, SGVPDRF from the VL CDR-2, or RDGAWFA from the VH CDR-3 of mAb II-481 produced 100–63% inhibition of binding between IgM RFs and the whole mAb II-481 confirmed the importance of these CDR epitopes reacting with the test RFs. Inhibition results using single or combinations of the CDR peptides reacting with RFs in free solution confirmed the importance of these portions of mAb II-481 CDRs as principal sites for RF binding in this idiotype–anti-idiotypic reaction.

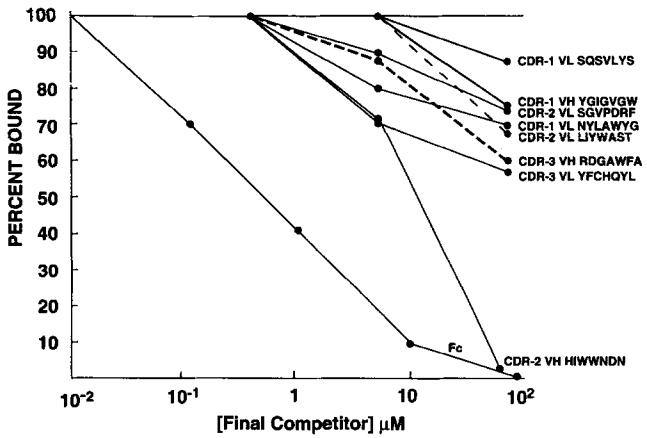


Figure 6. Competition/inhibition ELISA experiment in which AGER polyclonal IgM RF used at a concentration producing 50% maximum binding for mAb II-481 on the ELISA plate was preincubated with a broad range of concentrations of mAb II-481, RF-reactive heptapeptides. Maximum inhibition in this case occurred with CDR-2 VH peptide HIWWNDN and the positive control Fc of IgG. No inhibition was observed with control peptides such as the laminin peptide CIKVAVS.

Computer Modeling and Analysis of mAb II-481 RF-reactive Regions. A three-dimensional representation of the mAb II-481 was constructed using the previously established structures of mouse mAb D1.3 (29), B5.7 (30), and mAb A17 (31) as described in Materials and Methods. It was quite clear that major differences in conformation appeared within the Fv regions of the respective molecules (Fig. 7, *A* and *B*). The solvent-accessible, RF-reactive CDR regions of mAb II-481 were then highlighted on our model and are shown in Fig. 7 *C* with blue highlighting the H chain RF-reactive CDRs and yellow, the L chain RF-reactive CDR regions. An estimate of the actual shape of the mAb II-481 combining site was generated using Connolly solvent-accessible surfaces (33). This showed that the Fv region of mAb II-481 exhibited a characteristic Mexican hat (sombrero) appearance (Fig. 8 *A*). Because this shape very much resembled a prominent RF-reactive epitope on CH3 previously studied in some detail (36) (Fig. 8 *B*), direct comparisons of the topographical similarities of these two RF-reactive regions—the hypothetical mo-

Table 6. *Competition ELISA Results in Which Polyclonal IgM RF Were Preincubated with Combinations of mAb II-481 CDR Peptides before Completion of the ELISA with mAb II-481 on the Plate*

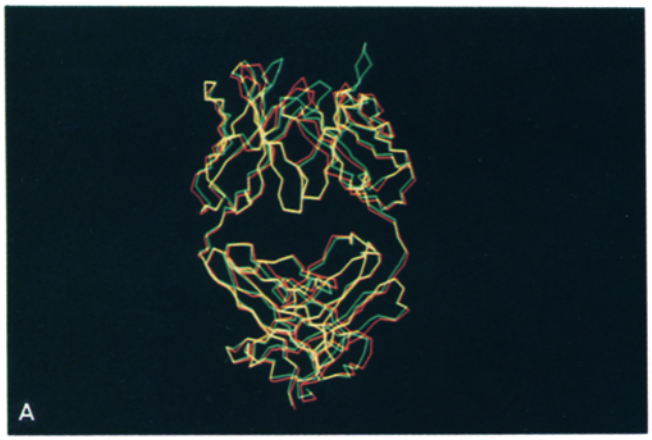
Combination of mAb II-481 CDR Peptides Tested in Inhibition	Polyclonal IgM RF tested							
	CLAU	AGER	LOP	STR	WEAK	MCC	SOR	ABR
CDR-1 VL NYLAWYQ (40)* Plus CDR-2 VH HIWWNDN (32)*	94‡							
CDR-1 VL NYLAWYQ (40) Plus CDR-2 VL LIYWAST (20) Plus CDR-2 VH HIWWNDN (32)	72							
CDR-1 VL NYLAWYQ (45) Plus CDR-3 VL YFCHQYL (44)		88						
CDR-1 VL NYLAWYQ (45) Plus CDR-3 VL YFCHQYL (44) Plus CDR-3 VH RDGAWFA (40)		96						
CDR-1 VL NYLAWYQ (20) Plus CDR-2 VL SGVPDRF (25) Plus CDR-3 VH RDGAWFA (45)			52					
CDR-1 VL NYLAWYQ (28) Plus CDR-3 VH RDGAWFA (63)				98				
CDR-1 VL NYLAWYQ (30) Plus CDR-2 VL SGVPDRF (72)					100			
CDR-1 VL NYLAWYQ (40) Plus CDR-2 VL SGVPDRF (80)						100		
CDR-1 VL NYLAWYQ (45) Plus CDR-2 VL SGVPDRF (90)							100	
CDR-1 VL NYLAWYQ (45) Plus CDR-1 VH YGIGVGW (40)							100	
CDR-2 VL SGVPDRF (90) Plus CDR-1 VH YGIGVGW (40)							100	
CDR-2 VL SGVPDRF (62) Plus CDR-2 VH HIWWNDN (80)								50
Lamin control peptide CIKVAVS§	0	0	0	0	0	0	0	0
Whole Fc human IgG	100	100	100	100	100	100	100	100

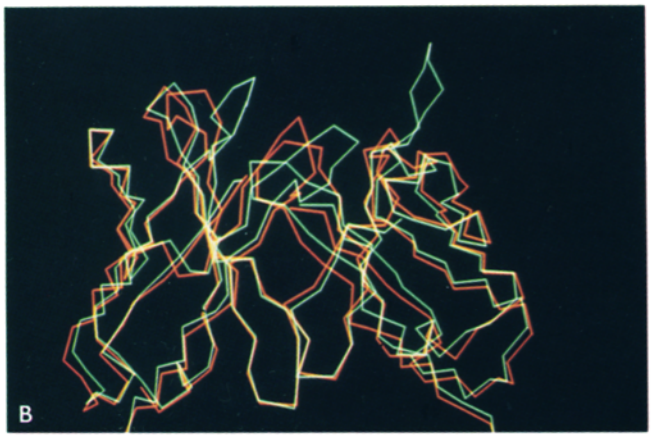
* Numbers in parentheses indicate inhibition by single peptide alone (see Table 5).
‡ Numbers indicate percent maximum inhibition with indicated combinations of CDR peptides tested over a broad micromolar concentration range.
§ Control peptides tested with RFs included CIKVAVS (laminin); GMERVRWCATDGEG (melanoma); LHNHYT (human $C_\gamma 3$); CNSRQTDREDELI (Klebsiella); ADAQTDREDLRTLLRY (HLA B27); AAEDWCKKGDT (IgA $C_\gamma 3$); and NSRQTDR (Klebsiella). None singly or in combination showed any inhibition of RF binding to mAb II-481.

deled structure of the Fv portion of mAb II-481 and the previously identified CH3 region—were made using the Sybyl computer graphics program. The remarkable apparent similarities in shape between these two different RF-reactive regions of entirely different antibody molecules are illustrated in Fig. 8 *C*. When 10 Å sections of the Fv RF-reactive region of mAb II-481 were examined (Fig. 8, *D* and *E*), preservation of the Mexican hat sombrero configuration was noted at many different levels. These comparisons supported a marked hypothetical conformational similarity between the two completely different RF-reactive Ig domains: the Fv portion of mAb II-481 and the CH3 region of human IgG.

Discussion

HSV induces Fcγ-binding proteins on infected cells and on virions. These Fcγ-binding proteins are coded for by the HSV genome and are able to bind the Fc part of human IgG





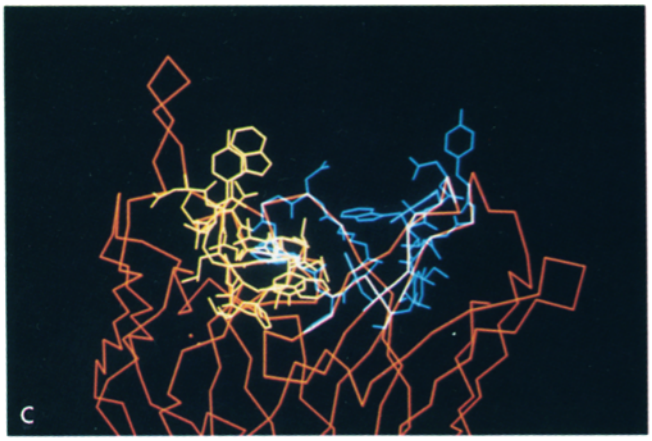
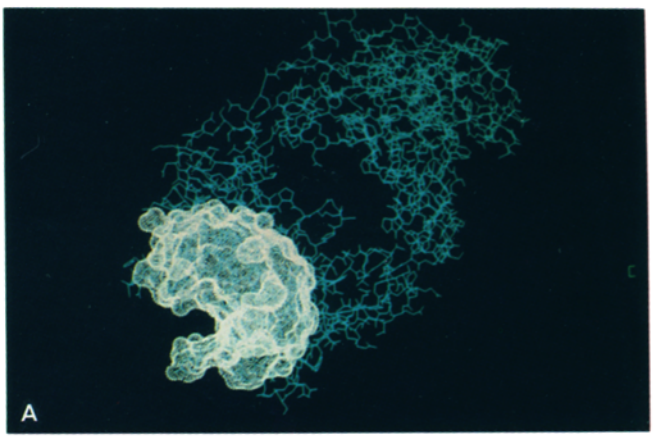
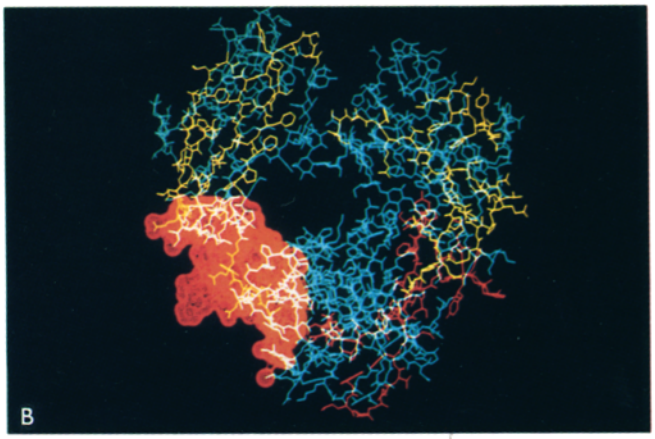


Figure 7. (*A*) Composite view as produced by Sybyl graphics of the template molecule (mouse mAb D1.3) used to produce the model of the Fab regions of mAb II-481 and the actual completed model of II-481. The template mAb D1.3 is shown in red and the modeled mAb II-481 in green and yellow. The upper portions containing the VL and VH regions are considerably different. (*B*) An enlarged view of the antibody combining sites of both molecules is shown in the two-color mode. The green/yellow carbon trace shows the modeled V regions of mAb II-481 and the red represents the same V regions of the template mAb D1.3. (*C*) Computer graphics scheme of Fv region of mAb II-481 showing RF-reactive residues within VL CDRs in yellow and RF-reactive residues within VH CDRs in blue. All RF-reactive residues shown in yellow or blue were judged to be solvent accessible.





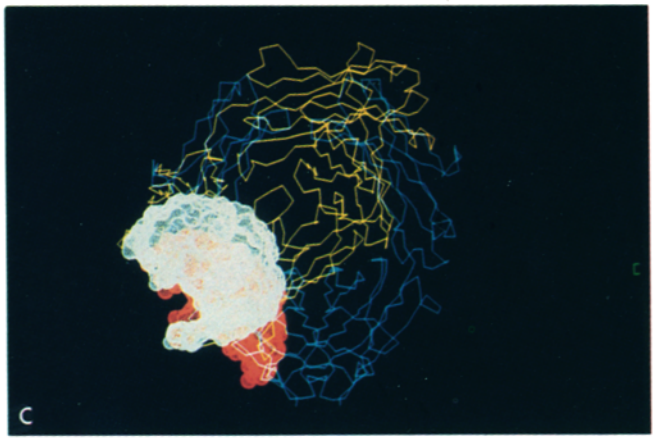
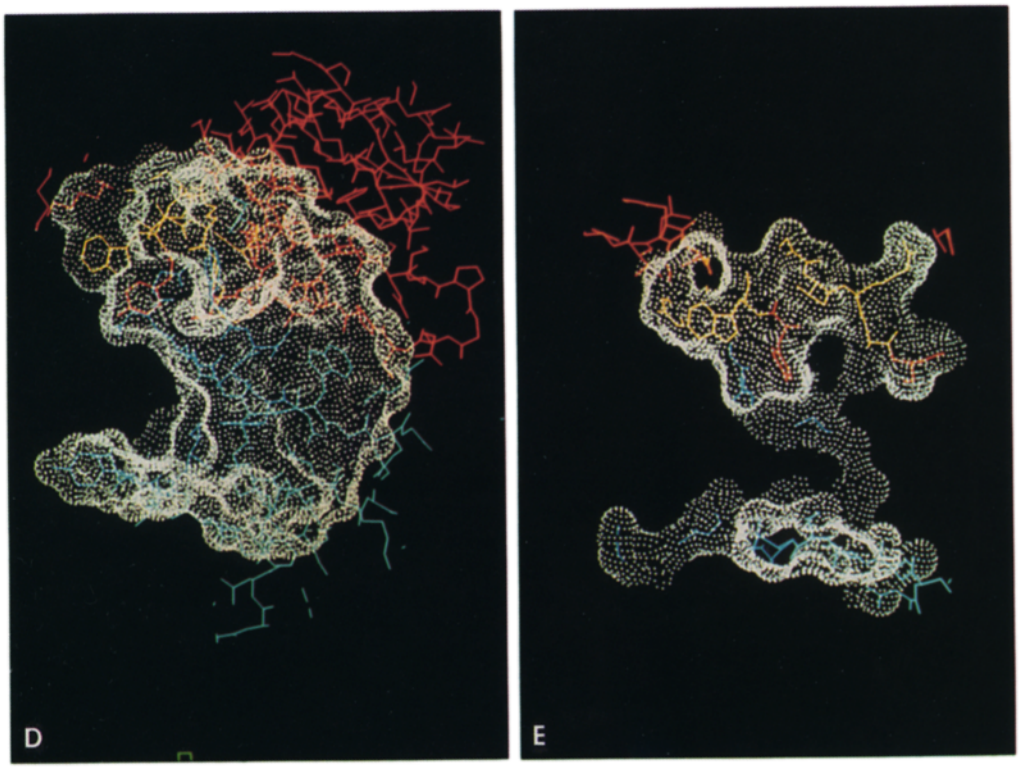


Figure 8. (*A*) Comparative Sybyl computer graphics views of the surface of the mAb II-481 combining site area (modeled in white showing what appears to be a Mexican hat configuration). The constant regions of the molecule shown in blue extend upward to the right. (*B*) By comparison, the surface modeling of a previously established CH3 RF-reactive region (EGLHNHY at residues 430–436 in CH3) is shown in red. The similarities in overall shape of the two RF-reactive IgG regions (one at the Fv portion of mAb II-481 in white) and the other at an outpouching of the IgG CH3 region (in *red*) are shown when the actual shapes of the two RF-reactive regions are overlaid. (*D* and *E*) Represents several 10 Å cuts through the Fv region of mAb II-481 showing variations of the shape at several levels.



(37–39). Mouse mAb II-481 reacts with the HSV-1 Fcγ-binding protein, gE, and the binding site of the mAb is directed at the Fc binding site on gE (14).

In the present study we have examined the reactions of human polyclonal and monoclonal IgM RFs with mouse mAb II-481 directed at the Fcγ-binding glycoprotein of HSV-1 as an example of an idiotype–anti-idiotype reaction. The opportunity to study this reaction was provided by our previous observations that many human RFs reacted with the Fab antigen-binding portions of mAbs to herpesvirus Fcγ-binding glycoproteins (12, 14). These findings suggested that some RFs could be generated as anti-idiotypic antibodies directed against HSV-1 Fcγ-binding proteins (11, 12, 13, 40) as shown in Fig. 9. The reaction between mAb II-481 and RF was inhibited by either IgG or highly purified gE (the Fcγ-binding herpes glycoprotein) (11, 12). Moreover, using an entirely different mAb (88-S) also directed against the Fcγ-binding protein of HSV-2, a similar RF specificity was demonstrated. Positive reactions were recorded for RF reacting with whole mAbs II-481 and 88-S and with their Fab but not their Fc fragments (12). These findings indicated that combining sites of many RFs may react with Fv regions of antibodies reacting with Fcγ-binding regions of the two herpesviruses HSV-1 and HSV-2.

If RFs from patients with RA react as anti-idiotypes against the Fv regions of mAb II-481, the antibody combining regions, therefore, the six (three VH, three VL) CDRs of mAb II-481 should contain antigenic epitopes very similar or identical to antigenic regions on CH3 and CH2 of human IgG reacting with human RF. Evidence supporting this latter hypothesis has been presented in the present report. The primary amino acid sequence of the VH and VL regions of mAb II-481 was established by PCR mRNA expansion and sequencing of the cDNA from the cell line producing the mAb. When the primary amino acid sequences of VH and VL mAb II-481 had been established, the entire solvent-accessible VH and VL regions of the latter were synthesized and tested as overlapping heptamers for RF reactivity. This approach showed

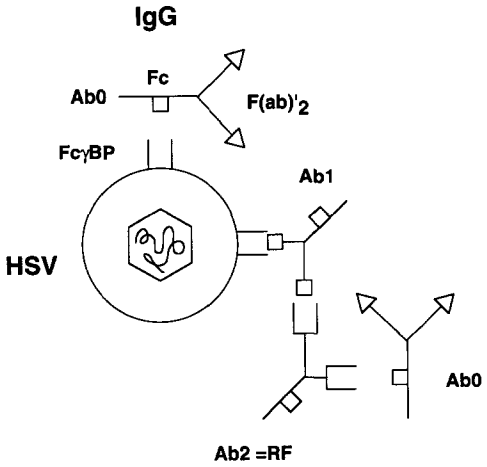


Figure 9. Anti-idiotype hypothesis regarding a conceivable association of viral Fcγ-binding protein and rheumatoid factor (*RF*). Viral Fcγ-binding protein on virions and on virus-infected cells are able to bind the Fc part of IgG (*Ab0*). RF might be produced as anti-idiotypic antibodies (*Ab2*) against antibodies (*Ab1*) directed towards the internal image of the viral Fcγ-binding proteins. The reactivity of most RFs is also directed against the Fc part of IgG (*Ab0*).

frequent localization of RF reactivity to the VH and VL CDR regions. Although each polyclonal or monoclonal RF displayed a slightly different reactive profile, the principal anti-mAb II-481 CDR reactivity of all RFs tested was clearly evident. No correlation was noted between anti-IgG subclass or anti-Gm specificity of RFs tested and their patterns of reactivity with VL or VH linear residues. It is recognized that testing RFs with linear heptamers of primary sequence is clearly not equivalent to assays of the whole Fv region since a number of antigenic epitopes on the VL and VH regions of mAb II-481 may be conformational and depend on three-dimensional structure. Nevertheless, the remarkable general overall patterns of reactivity with small solvent-accessible linear regions of the CDRs suggest that they may contribute to important reactive sites for RFs. Further strong evidence that the linear regions within the three mAb II-481 CDRs contributed important reactive epitopes for test RFs was provided by the CDR peptide inhibition data summarized in Tables 5 and 6. For each RF, a single CDR peptide produced 63–100% inhibition of binding between test RFs and whole mAb II-481 on the ELISA plate. With the panels of RFs tested, some single CDR peptides or combinations of CDR peptides inhibited RF binding to mAb II-481 better than others, although many RFs had reacted with most VL or VH CDR regions in the pin ELISA assay. It seems possible that CDR peptides showing the greatest inhibition actually represented portions of the antigenic sites of II-481 bearing the greatest avidity for the particular RF being tested. These data emphasized that the major peaks of RF reactivity within the three CDRs of mAb II-481 synthesized as linear overlapping heptamers on the pins were real phenomena and could be confirmed by inhibition of RF reactivity using preincubation of single CDR peptides with IgM RFs in free solution. Moreover, increases in RF inhibition to 100%, noted when several combinations of II-481 CDR peptides were employed together, further support the significance of their individual contributions to RF antigen-binding sites. Since the use of single or combinations of CDR peptides could never exactly approximate the three-dimensional conformation of the mAb II-481 combining site, it often required much higher micromolar concentrations of CDR peptide than Fc of IgG or mAb II-481 for complete inhibition. This was to be expected since the affinity between RF and single or several peptides could never exactly reproduce that with the whole antibody Fv portion. A complete replication of the three-dimensional configuration of mAb II-481 CDRs cannot, of course, be hoped for with the single linear peptides tested, however, the results with peptide inhibition demonstrated that this approach could provide remarkably detailed information concerning RF-reactive epitopes.

Finally, the three-dimensional shape or conformation of the Fv portion of mAb II-481 was modeled using computer graphics and coordinates established for several template mAbs previously studied. It must be emphasized that our three-dimensional model of mAb II-481 is still theoretical and will require confirmation when gE and Fab II-481 have been crystallized and studied for their exact three-dimensional structure. However, when the spacial distribution and shape of the Fv portion of mAb II-481 was estimated using approximation of the areas of the individual atoms involved, a sombrero conformation was found. This particular shape bore a striking resemblance to a previously identified CH3 epitope showing strong RF reactivity (36). This latter epitope was based on established three-dimensional CH3 structure according to Diesenhoffer et al. (41, 42). Of interest was the fact that the CH3 epitope visualized in its three-dimensional conformation showed such a striking homology of shape with the theoretical model of the Fv region of mAb II-481 constructed using our modeling procedure. This region contained the two histidines at positions 433 and 435 and the tyrosine at 436, which had previously been identified as important epitopes for RF reactivity (43). Although an exact homologous sequence to the EGLHNHY at this region (residues 430–436 in CH3) was not present in the Fv region of mAb II-481, it was clear that the two regions shared many similarities in conformation. Thus a number of separate lines of evidence presented in the present report provide intimate insight into precise residues within the Fv of mAb II-481 idiotype reacting with RFs in an idiotype–anti-Id reaction. The primary reactivity of the polyclonal and monoclonal RF with the CDR-1, -2, and -3 regions confirmed that such RFs react with regions contributing to the antigen binding sites of mAb II-481 directed against the Fcγ-binding protein of HSV-1. Data presented here also provide a detailed structural basis for the idiotypic–anti-idiotypic reactivity of human RFs with the Fab fragment of mAb II-481.

Additional support for a structural resemblance between some RFs and the HSV-Fcγ-binding protein lies in the striking similarities shown in their IgG binding specificities. Polyclonal RFs derived from patients with RA generally possess a similar human IgG subclass specificity to that of HSV-1 Fcγ-binding protein in that they preferentially bind to IgG-1, -2 and -4 but not or only weakly to IgG-3 (43, 44). Like the HSV Fcγ-binding proteins, most polyclonal RFs bind to the CH3 and CH2 interface region of IgG (43, 45–47). Studies using chemical modification of amino acids of IgG revealed that Tyr and His residues on IgG seemed to be critical for the binding with RFs (38, 40, 45, 48) and also for binding to the HSV Fcγ-binding protein (47).

As an alternative to the idiotype–anti-idiotype model, it is conceivable that the viral herpes Fcγ-binding protein gE may present the IgG molecule to the host (patients with RA) in such a way that the interface regions between the IgG CH2 and CH3 domains become immunogenic (40). RF-positive patients with RA have been demonstrated to show an association with the class II HLA antigen DR4 (49, 50). It is possible that HLA DR4 could somehow influence the immune response against Fcγ-binding proteins such as gE or to autologous anti-Fcγ–binding protein antibodies and that RFs in RA might in turn, therefore, be related somehow to immune responses of genetically predisposed individuals to herpesviruses or to other viral agents possessing IgG Fcγ-binding proteins. Presently, there is no evidence that HLA DR4 functions as a restriction element for HSV-1 immune responsiveness.

Production of RF cannot be ascribed to idiotype–anti-

idiotypic responses alone since there is now a large body of experimental and clinical evidence which indicates that immunization with globular proteins, certain carbohydrates, or bacterial antigens may also result in RF induction.

The information provided here concerning the reaction between polyclonal or monoclonal IgM human RFs and the Fv portions of mAb II-481 can be compared with the precise three-dimensional definition of an idiotope–anti-idiotope complex determined from the crystal structure of a complex between the monoclonal antilysozyme Fab D1.3 and the anti-idiotopic Fab E225 at 2.5 Å resolution (51). In this latter instance, the private idiotope consisted of 13 amino acid residues mainly from the CDRs of D1.3. 7 of the 13 residues made contacts with the original antigen which indicated a significant overlap between idiotype and antigen-combining site. In the case of mAb II-481 studied here, we do not yet have final definition of actual gE antigen binding residues within the mAb II-481 VH or VL CDRs. When this information becomes available with a crystal structure for the gE–mAb II-481 complex, a more selective or precise estimate of idiotope and antigen combining site may become available.

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