DELINEATION OF A CROSS-REACTIVE IDIOTYPE ON HUMAN AUTOANTIBODIES WITH ANTIBODY AGAINST A SYNTHETIC PEPTIDE

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The molecular basis of immunoglobulin idiotypes (1) has been pursued in several well-defined antibody systems (2–7). The accumulated results have suggested that the hypervariable regions (complementarity-determining regions, CDR)[1] of the light and heavy polypeptide chains usually contribute to the formation of idiotypic determinants. In the extensively analyzed murine anti-dextran model, one private idiotype and one public idiotype (cross-reactive idiotypes, CRI) were assigned, respectively to the third and the second hypervariable regions of the heavy chain (6). In most other cases, idiotypic determinants have not been associated definitively with a specific amino acid sequence (2). The latter result is not surprising, considering that antiidiotype antibodies elicited by immunization with intact immunoglobulin often recognize determinants dependent upon a particular quaternary interaction of the light and heavy chains (2).

Using carefully absorbed rabbit antisera, Kunkel and colleagues (8) first described the unusual existence of two major CRI (Wa and Po) among human monoclonal IgM anti-IgG autoantibodies (rheumatoid factors, RF) from unrelated individuals with cryoglobulinemia. The larger Wa group includes ~60% of monolonal IgM-RF (8, 9). Amino acid sequence analysis of the variable regions of two Wa-CRI positive IgM-RF (Sie and Wol) has revealed a marked homology between the kappa light chains, but not the heavy chains (10). However, the precise chemical nature of the Wa-CRI has remained unclear.

To clarify these issues, we generated a murine monoclonal antibody (mAb 17–109) that recognized a CRI shared by the Wa-CRI positive IgM-RF Sie and Glo but not by the Wa-CRI negative IgM-RF Lay and Pom (11). Although made

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[1] *Abbreviations used in this paper:* BBS, borate-buffered saline; BSA, bovine serum albumin; CDR, complementarity-determining regions; CRI, cross-reactive idiotypes; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PSH3, synthetic *peptide* corresponding to the *3rd* CDR of the *heavy* chain of the IgM-RF *Sie*; PSL2, synthetic *peptide* corresponding to the *2nd* CDR and adjacent residues on the *light* chain of the IgM-RF *Sie*; RF, rheumatoid factors.

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against the intact IgM-RF Sie protein, the mAb 17–109 also reacted with isolated kappa chains from Wa-CRI positive IgM-RFs. A comparison of the reported amino acid sequences of the kappa chains of the Wa-CRI positive IgM-RF indicated that among the three CDR, only the second CDR was identical (reference 10 and Table I).

Antibodies against synthetic peptides have been able to identify with precision predefined antigenic determinants in intact proteins (12–14). Recently, we prepared an antiidiotype against a "private," or individually specific idiotypic determinant on IgM-RF Sie, by immunization with a synthetic peptide corresponding to the third CDR of the heavy chain (15). It seems possible that a similar strategy would enable us to determine the relationship between the CDR shared in common by human IgM-RF, and the CRI-Wa. The current communication demonstrates the success of this approach in delineating the structural basis of a CRI on human autoantibodies.

Materials and Methods

Preparation of the Synthetic Peptide. A peptide, designated PSL2, was synthesized that corresponded to amino acid residues 49–61 (second CDR and its neighbors, Table I; 16) in the light chain of the monoclonal IgM-RF Sie, according to the sequence reported by Andrews and Capra (10). It was composed of tyr-gly-ala-ser-ser-arg-ala-thr-gly-ile-pro-asp-arg. An additional cys was added to the C-terminal end of the sequence to facilitate coupling to the protein carrier (15).

The PSL2 was prepared by the solid-phase method (17), slightly modified as previously described (15, 18). Briefly, *N*-tert-butoxy-carbonyl-tyrosine (BOC-tyr)-resin was used along with the following side chain protecting groups: (O-bromo-benzyoxycarbonyl) for tyrosine; O-benzyl for threonine, serine, and aspartic acid; S-methoxybenzyl for cysteine; tosyl for arginine. All couplings used a 10-fold excess of protected amino acid plus dicylohexylcarbodiimide. The reactions were 99% complete by the picric acid test. At the end, the peptide polymer was treated with anhydrous hydrogen fluoride. The cleaved peptide was extracted with anhydrous ether. The amino acid analysis of the peptide was within 5% of theory.

The synthesis of the control PSH3 (corresponding to the third CDR of the H chain of the IgM-RF Sie) has been described previously (15). It was composed of glu-trp-lys-gly-gln-val-asn-val-asn-pro-phe-asp-tyr-gly-gly-cys.

TABLE I

Amino Acid Sequences of the Kappa Chains of Human Monoclonal IgM-RF Paraproteins in the Second CDR and Adjacent Framework Region

IgM-RF	CRI‡	Region*:	FR-2	CDR-2							FR-3				
		Residue*:	49	50	51	52	53	54	55	56	57	58	59	60	61
1. Sie§	Wa		Tyr	Gly	Ala	Ser	Ser	Arg	Ala	Thr	Gly	Ile	Pro	Asp	Arg
2. Wol§	Wa		——	——	——	——	——	——	——	——	——	——	——	——	——
3. Pom‖	Po		——	——	——	——	Thr	——	——	——	——	——	——	Ala	——
4. Lay‖	Po		——	——	——	——	Thr	——	Glu	Ala	——	Val	——	Ser	——
5. Got¶	NA		——	——	——	——	——	——	——	——	——	——	——	——	——
6. Neu¶	NA		——	——	——	——	——	——	——	——	——	——	——	——	——
7. Pay¶	NA		——	——	——	——	——	——	——	——	——	——	——	——	——
8. Gar¶	NA		——	——	——	——	——	——	——	——	——				

* The region and the residue number were assigned by Kabat (16). *FR*, framework region.

‡ The cross-reactive idiotypes (CRI) were determined by Kunkel et al. (8). *NA*, idiotype not assigned.

§ Amino acid sequences were reported by Andrews and Capra (10).

‖ Amino acid sequences were reported by Klapper and Capra (5).

¶ Amino acid sequences were reported by Ledford et al. (21).

Immunization of Animals. The synthetic peptide PSL2 was conjugated via its terminal cysteine to keyhole limpet hemocyanin (KLH) with M-maleimidobenzoyl *N*-hydroxysuccinamide ester, as described previously (15, 19). Each of three rabbits was injected subcutaneously with 2.5 mg of the conjugate, emulsified in complete Freund's adjuvant. The injection was repeated two months later. 3 wk after the second immunization, the rabbits were boosted again with 2.5 mg glutaraldehyde cross-linked peptide PSL2 in incomplete Freund's adjuvant. The latter reagent was prepared by the addition of glutaraldehyde (final concentration 0.25% vol/vol) to a 5 mg/ml solution of peptide in isotonic phosphate-buffered saline (PBS), followed by 1 h incubation at room temperature, and immediate suspension in adjuvant. Thereafter, the rabbits were bled, and the sera was stored at 20°C until analyzed.

Purification of Proteins. Plasma, purified proteins, or purified light chains (of IgM-RF Got) from patients with monoclonal IgM cryoglobulins were kindly donated by Drs. J. D. Capra, G. Abraham, F. Goni, B. Frangione, J. Johnson, and H. Metzger. The IgM cryoglobulins were purified by repeated precipitation at 4°C, followed by chromatography on Sephadex G-200 or Ulltrogel AcA 22 in 0.2 M sodium acetate, pH 3.5. IgM and IgG peaks were identified by immunodiffusion. The appropriate fractions were pooled and stored at −20°C. Pooled human IgG was prepared from Cohn fraction II (Sigma Chemical Co., St. Louis, MO) by DEAE cellulose chromatography in 0.01 M sodium phosphate, pH 8.0. Pooled polyclonal human IgM was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

The heavy and light chains of the IgM-RF proteins Sie and Glo were separated on a Sephadex G-100 column with 1 M acetic acid, or a TSK-250 column (Bio-Rad Laboratories, Richmond, CA) with 1 M propanoic acid, after complete reduction and alkylation (20). The polypeptides were stored frozen at 1 mg/ml. Using a radioimmunoassay specific for IgM heavy chains or kappa chains, we estimated that the heavy chains contained not more than 5% light chains, while the light chains contained not more than 2% heavy chains.

Enzyme-linked Immunosorbent Assay (ELISA). Either synthetic peptides (100 or 2 μg/ml), various purified monoclonal IgM-RF (10 or 2 μg/ml), isolated light chains from the IgM-RF proteins (10 or 2 μg/ml), affinity purified anti-peptide antibody (8 μg/ml), or antibody-depleted rabbit IgG (8 μg/ml) were each dissolved in 0.1 M borate, 0.2 M NaCl, pH 8.2 (BBS), and were added to plastic microtiter plates (Costar #3590) at 100 μl/well. After an overnight incubation at 4°C, the plates were washed twice with BBS contianing 0.5% Tween-20 (Sigma #P-1379, BBS/Tween-20) and were quenched with BBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Then, 100 μl of various samples diluted in BBS containing 0.5% BSA were distributed to duplicate wells. After incubation for 3 h, the plates were washed with BBS/Tween-20. Aliquots of a 1:500 dilution in PBS of human IgG-adsorbed, alkaline phosphatase–labeled, goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD), were dispensed to the wells. The plates were incubated for 1 h at room temperature. After washing, *p*-nitrophenyl phosphate (1 mg/ml) in 0.05 M sodium carbonate, pH 9.8 was added to each well. After 1–16 h incubation, the absorption at A_{405} was measured. Inhibition of the anti-peptide antibody binding to plates coated with IgM-RF or the isolated light chains of IgM-RF was assessed similarly, except that the anti-PSL2 antiserum (at 1:100 dilution) was first mixed with an equal volume of the different inhibitors.

To assess the binding of liquid phase IgM-RF Sie to coated anti-PSL2 antibody, bovine intestine alkaline phosphatase (Sigma Chemical Co.) was first coupled to IgM-RF Sie according to the manufacturer's instructions. Then, the standard ELISA method was modified as follows. 75-μl aliquots of the PSL2 peptide, the PSH3 control peptide, or buffer alone were added to wells precoated with anti-peptide antibodies or antibody-depleted IgG. After 1 h incubation at room temperature, 75 μl of enzyme-conjugated IgM-RF Sie was distributed to the wells. The plate was incubated 1.5 h at room temperature, and enzyme activity was measured.

Adsorption and Elution of the IgM-RF Sie-binding Activity. The globulins (100 mg) from 8 ml of anti-peptide antiserum were precipitated with 33% saturated ammonium sulfate,

and redissolved in 10 ml of BBS. The solution was passed through a BSA-column to remove nonspecific binding activity, and then was recirculated over a PSL2-coupled Sepharose 4B affinity column (ca. 1.6 mg/ml gel × 3 ml), that had been prepared with cyanogen bromide activated Sepharose-4B (Sigma Chemical Co.). After removal of non-bound material with BBS, the anti-peptide antibody was eluted with 0.1 M glycine HCl, pH 2.5, and then was dialyzed against BBS.

Results

Induction of Anti-RF Antibody with the PSL2 Peptide. After receiving two subcutaneous injections of the PSL2 peptide conjugated to KLH and one injection of glutaraldehyde cross-linked peptide, the three rabbits were bled, and their sera were analyzed for anti-peptide antibody activity by the ELISA method. As shown in Table II, all three antisera contained high titers of anti-PSL2 antibodies. Notably, the three antisera also reacted with plates coated with intact IgM-RF Sie. Under the same conditions pooled sera from normal rabbits did not bind significantly to plates coated with PSL2 or IgM-RF Sie. In addition, the anti-PSL2 immune sera did not react with plates coated with the control peptide PSH3, or the Wa-CRI negative IgM-RF Lay.

Association of Anti-RF Antibody Activity with the PSL2 Peptide. Three different experiments showed that the anti-RF antibody induced by the PSL2 peptide recognized a specific PSL2 peptide-determined antigen. First, as shown in Table II, experiment 2, the antisera reacted with the isolated light chain, but not the heavy chain, of the IgM-RF Sie. Second, the binding of the anti-PSL2 antibody to both intact IgM-RF Sie and to the isolated light chain of Sie (Table III) was completely inhibited by the free PSL2 peptide, but not by the control PSH3

TABLE II

*Induction of Anti-RF Antibodies with the PSL2 Peptide**

Experiment	Rabbit antiserum	Antigen: (A_{405} × 10^3)					
		Peptide		IgM-RF		IgM-RF-Sie	
		PSL2	PSH3	Sie	Lay	Light chains	Heavy chains
1.‡	Normal	23	25	35	28	—**	16
	Anti-PSL2 (1)‖	1,036	9	136	22	—	9
	Anti-PSL2 (2)	948	24	92	19	—	13
	Anti-IgM	15	38	1,602	1,453	—	962
2.§	Normal	6	7	9	7	14	14
	Anti-PSL2 (1)	430	14	64	7	228	14
	Anti-PSL2 (3)	489	18	110	17	420	23
	Anti-IgM	0	1	637	441	378	525
	Anti-PSH3¶	6	668	259	0	30	510

* Anti-peptide antisera were assayed at 1:1,000 dilution, and anti-IgM antisera at 1:10,000 dilution. Bound antibodies were detected with alkaline phosphatase–conjugated goat anti-rabbit IgG and substrate. The numbers represent A_{405} measured after 1 h incubation at room temperature.

‡ Antigens were coated at either 100 μg/ml (peptides) or 10 μg/ml (IgM-RF and isolated chains).

§ Antigens were coated at 2 μg/ml.

‖ The numbers in parentheses designate the three rabbit anti-PSL2 antisera.

¶ Antiserum from a rabbit immunized with the control peptide PSH3 (15).

** Not done.

TABLE III

*Inhibition of Anti-PSL2 Binding to IgM-RF by the Free PSL2 Peptide**

IgM-RF	Percent inhibition‡ peptide:					
	PSL2			PSH3		
	50	500	5,000	50	500	5,000
	ng/ml					
Sie	29	85	100	0	0	0
Sie kappa chains	93	96	97	5	5	5
Glo kappa chains	69	100	100	10	5	4
Gar	70	81	80	7	0	0
Got	92	96	99	5	14	4
Neu	87	96	98	1	0	3
Pay	89	92	90	9	6	0
Got-L	84	93	95	2	6	1

* Increasing amounts of peptide inhibitors were added to anti-PSL2 antiserum 1:100 in BBS before distribution to wells previously coated with the indicated IgM-RF or the isolated light chains of IgM-RF. The A_{405} was measured 1 h after the addition of substrate to the wells.

‡ Percent inhibition = 100 − 100 × (Anti-PSL2 antiserum with inhibitors − Normal rabbit serum)/(Anti-PSL2 antiserum − Normal rabbit serum).

TABLE IV

Adsorption and Elution of the Anti-RF Activity from a PSL2-Coupled Affinity Column

Antibody	Antigen coated ($A_{405} \times 10^3$)		
	BSA	Peptide	IgM-RF Sie
Original, 10 μg/ml	24	1,319	303
BSA adsorbed, 10 μg/ml	0	1,214	250
BSA and PSL2 adsorbed, 10 μg/ml	0	38	25
PSL2 eluate 10 μg/ml	12	1,859	730
PSL2 eluate (1 μg/ml)	0	1,616	375

An IgG fraction (100 mg) of anti-PSL2 antiserum in 10 ml BBS was first adsorbed with a 3-ml BSA-agarose column (5 mg/ml gel), and then was loaded onto a 3-ml PSL2-coupled column (1.6 mg/ml gel). After 15 min incubation at room temperature, the effluent was collected. After washing with BBS, the bound material was eluted with 0.1 M glycine-HCl, pH 2.5 and was dialyzed into BBS. The ELISA was done as described in Table II.

peptide. Third, the anti-Sie antibody activity was adsorbed by, and eluted from a PSL2-coupled Sepharose affinity column (Table IV).

Defining a CRI on Human IgM-RF with Anti-PSL2 Antibodies. Among monoclonal IgM-RF, affinity-purified anti-PSL2 antibody reacted strongly with Sie and Glo, weakly with Pom, and not at all with Lay (Table V, Experiments 1 and 2). Under the same conditions, the anti-PSL2 antibody did not react significantly with pooled human IgM and IgG. Furthermore, the anti-PSL2 antibody reacted with the isolated kappa chains of IgM-RF Glo. The binding was inhibited by the free PSL2 peptide, but not by the control PSH3 peptide (Table III).

Subsequent to the completion of the above experiments, Ledford and co-

TABLE V

Reactivity of Affinity-purified Anti-PSL2 Antibody with Human Monoclonal IgM-RF

Experiment	Antigen coated[‖]	$A_{405} \times 10^3$* Sample			
		Anti-PSL2 enriched	Anti-PSL2 depleted	Specific binding	Anti-IgM[‡]
1.	Sie	1,388	399	989	ND
	Pom	549	349	200	ND
	Lay	50	16	34	ND
2.	Sie	1,545	144	1,401	ND
	Glo	>1,999	686	>1,313	ND
	Lay	57	31	26	ND
	Pooled IgM	61	35	26	ND
	Pooled IgG	60	27	33	ND
3.	Sie	456	57	399	912
	Glo	603	126	477	695
	Lay	19	13	6	781
	Pooled IgM	33	15	18	624
	Pooled IgG	26	8	18	237
	Got	505	3	502	25
	Neu	1,262	4	1,258	171
	Pay	567	1	566	56
4.	Sie	1,861	177	1,684	ND
	Neu	564	22	542	ND
	Gar	508	21	487	ND

* A_{405} was measured after overnight incubation at room temperature.

‡ Anti-PSL2–enriched and –depleted IgG are the eluate and the peptide adsorbed fractions described in Table IV. Each was used at 1 μg/ml. Anti-IgM antiserum was diluted 1:10,000. *ND*, not done.

§ Specific binding = A_{405} of enriched anti-PSL2 antibody − A_{405} of antibody-depleted IgG.

‖ Antigens were coated at 2 μg/ml.

workers (21) reported several new sequences of monoclonal IgM-RF kappa chains. These investigators kindly made available to us some of the proteins. As shown in Table I, the amino acid sequences of the kappa chains of the Got, Neu, Pay, and Gar proteins are the same as Sie in the PSL2-associated region. Table V (Experiments 3 and 4) shows that each of the four different IgM-RF, as well as the isolated light chain of IgM-RF Got, was identified by affinity-purified anti-PSL2 antibody. Once again the binding was inhibited specifically by the free PSL2 peptide (Table III).

Binding of IgM-RF in Solution to Anti-PSL2 Antibody. Conceivably, the coating of IgM-RF onto plastic wells might denature the immunoglobulin sufficiently to allow exposure of a CDR-2 associated idiotypic antigen that was not expressed by intact IgM, or by free kappa chains. To explore this possibility, we tested the binding of IgM-RF Sie in solution to affinity-purified anti-PSL2 antibody. As shown in Fig. 1, alkaline phosphatase–labeled IgM-RF Sie in solution bound directly to anti-PSL2 antibody-coated plates. The reaction was inhibited by the free PSL2 peptide.

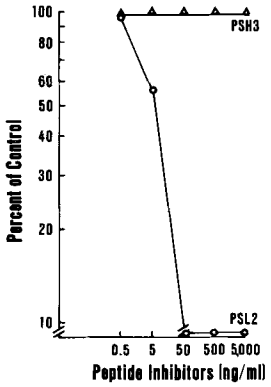

FIGURE 1. Inhibition by free peptide of IgM-RF Sie binding to anti-peptide coated plates. Increasing amounts of peptides PSL2 (○) or PSH3 (△) were added to wells precoated with affinity-purified anti-PSL2 antibody. After 1 h incubation at room temperature, enzyme-labeled IgM-RF Sie (10 μg/ml) was added to each well. 90 min later, the plate was washed, and A_{405} was measured 1 h after the addition of substrate to the wells.

Discussion

The present experiments describe the antiidiotypic properties of antibody against a defined hypervariable region peptide shared by many human IgM-RF autoantibodies. The antibody was generated by immunization of rabbits with synthetic peptide (PSL2) corresponding to the second CDR and adjacent residues on the kappa chain of the monoclonal human IgM-RF Sie. The antibody reacted efficiently with (*a*) the intact IgM-RF Sie protein, (*b*) the IgM-RF Glo, which shares a kappa chain associated CRI with IgM-RF Sie (11), and (*c*) four recently described monoclonal IgM-RF paraproteins (Neu, Gar, Pay, and Got), whose kappa chains include the PSL2 sequence (21). In addition, the antibody cross-reacted weakly with IgM-RF Pom, whose kappa chains differ from IgM-RF Sie by two residues in the PSL2 region. The antibody did not react with pooled human IgM and IgG, nor with IgM-RF Lay, whose kappa chains differ from IgM-RF Sie by five amino acid residues in the PSL2 region. The anti-PSL2 antibody recognized the isolated kappa chains of the IgM-RF paraproteins Sie, Glo, and Got, but did not bind the isolated Sie and Glo heavy chains. The binding of the antiserum to all reactive IgM-RF and to the light chains of Sie, Glo, and Got, was completely inhibited by the free PSL2 peptide, but not by a control peptide, PSH3. Collectively, these results strongly suggest that the synthetic PSL2 peptide induced an antiidiotype against a CRI associated with the kappa light chains of human IgM-RF autoantibodies. The molecular basis of this CRI is delineated by the PSL2 amino acid sequence.

The classic studies of Kunkel and co-workers (8) originally demonstrated similarities in the light chains of IgM-RF paraproteins showing cross-idiotypic specificities. These investigators discovered that for the majority of IgM-RF, the light chains were kappa in type, kappa III in subgroup, and kappa IIIb in sub-subgroup (9). They further suggested that perhaps a kappa IIIb′ sub-sub-subgroup accounted for the observed cross-idiotypy. The present association of a CRI on human IgM-RF paraproteins with a predefined sequence represented

by the PSL2 peptide is a logical outcome of these earlier predictions. The delineation of a specific peptide-defined antigen in the variable regions of the light chains of human IgM-RF coalesces the former distinction between idiotype, variable region allotype and sub-sub-subgroup. An important objective of future studies will be to determine the incidence of the same CDR sequence among RF and non-RF kappa chains in families and populations. Eventually, one must also ascertain whether or not the related DNA sequence is represented in the germ line kappa V-region pool of patients with idiotype-positive proteins. The availability of oligonucleotide probes corresponding to the PSL2 peptide would facilitate such studies.

Family studies have shown that the individually specific idiotypes on IgM-RF autoantibodies may be inherited (22). However, the exact genetic interpretation of the serological data has remained unclear, because the full expression of the RF idiotype depended on both the light chains and heavy chains of Ig, which are encoded by genes on separate chromosomes. The identical problems have hampered serological investigations of idiotypic inheritance in animal systems. In future studies, antibodies of predefined specificity against several different CDR and framework regions in the RF molecule may enable us to ascertain the individual role of light and heavy chain variable regions in the inheritance of IgM-RF idiotypes (8, 22–27).

Kabat et al. and Kindt and Capra (28–30) have proposed that the variable region genes of immunoglobulin are assembled from small segments of DNA "minigenes." One experimental approach to test the minigene/gene insertion theory is to analyze idiotype-bearing antibodies in animals suppressed with antibodies against adjacent framework regions (29). Experiments of this type require antiidiotype reagents that delineate relatively short primary structures in the variable regions of light and heavy chains. A similar experimental approach would help assess the role of gene conversion in immunoglobulin diversification. Antibodies against synthetic peptides should facilitate a serological analysis of the possible role of both gene insertion and gene conversion (31, 32) in the generation of antibody diversity. The serological results could then be confirmed by direct protein and DNA sequence analysis.

Summary

Antibody against a cross-reactive idiotype (CRI) on human IgM-rheumatoid factor (RF) antibodies was induced by immunization of rabbits with a synthetic peptide (PSL2) corresponding to the second complementarity-determining region (CDR), and adjacent amino acid residues of the kappa light chain of the IgM-RF Sie. The anti-peptide antibody bound efficiently to IgM-RF proteins known to share a cross-reactive idiotype, and to their isolated kappa chains. The anti-CRI was absorbed by, and eluted from, a peptide-Sepharose affinity column. The antibody activity was inhibited by the free peptide in solution. The anti-peptide antibody thus identifies a public idiotype on human IgM-RF, that is largely dependent on the primary sequence of the second CDR of the light chain. Such peptide-induced antiidiotypes of predefined specificity may facilitate studies of the molecular basis of idiotypic cross-reactions, the inheritance and somatic diversification of antibody molecules, and the regulation of the idiotype network.

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