CHARACTERIZATION OF HUMAN RHEUMATOID FACTORS WITH SEVEN ANTIIDIOTYPES INDUCED BY SYNTHETIC HYPERVARIABLE REGION PEPTIDES

BY POJEN P. CHEN,* FERNANDO GÕNI,§ RICHARD A. HOUGHTEN,‡ SHERMAN FONG,* ROBERT GOLDFIEN,* JOHN H. VAUGHAN,* BLAS FRANGIONE,§ AND DENNIS A. CARSON*

*From the Departments of *Basic and Clinical Research, and ‡Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037, and the §Department of Medicine and Pathology, New York University Medical Center, New York 10016*

Rheumatoid factor (RF)[1] autoantibodies against autologous IgG are found in the sera of most patients with rheumatoid arthritis (RA) (1). IgM-RF, and particularly IgG-RF comprise a large part of the immune complexes that are abundant in rheumatoid synovial fluid, and may contribute to complement consumption and chronic tissue damage (2).

RF are not specific for RA. The autoantibodies have been detected in the sera of some normal individuals (3) and in the culture supernatants of polyclonally activated normal lymphocytes (4). IgM-RF autoantibodies are produced regularly during primary and secondary immune responses in humans and mice (5–7). In a well-defined murine system, IgM-RF synthesis was dependent upon both immune spleen cells and the relevant antibody (8, 9). These results strongly suggest that IgM-RF are a physiologic component of the immune system (7, 10).

Conceivably, antibodies with evolutionarily significant protective or regulatory functions may be encoded by germ line V_H and V_L (variable heavy and light chains) genes. Crossreactive idiotypes (CRI) represent serologic markers for Ig V_H and V_L sequences. In elegant studies, Kunkel and coworkers (11) described two CRI, (Wa and Po), on Waldenstrom's macroglobulins with IgM-RF activity. The mutually exclusive Wa and Po CRI were expressed, respectively, by 65% and 20% of monoclonal IgM-RF. Amino acid sequence analyses (12, 13) revealed that Wa-CRI$^+$ IgM-RF have similar L chains, and that Po-CRI$^+$ proteins have similar H chains.

To gain insight concerning structural and genetic relationships among a larger series of IgM-RF proteins in an outbred human population, we generated two

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[1] *Abbreviations used in this paper:* BBS, borate-buffered saline; BSA, bovine serum albumin; CDR, complementarity-determining region; CFA, complete Freund's adjuvant; CRI, crossreactive idiotype; D, diversity region of Ig; ELISA, enzyme-linked immunosorbent assay; H, heavy chain of Ig; J, joining region of Ig; KLH, keyhole limpet hemocyanin; L, light chain of Ig; RA, rheumatoid arthritis; RF, rheumatoid factor; V, variable region of Ig.

different antiidiotypic antibodies against short, primary sequence–dependent idiotypic determinants on RF H and L chains (14, 15). In initial experiments, an antiidiotypic antibody was induced by immunization with a synthetic peptide corresponding to the H chain third hypervariable region (complementarity determining region) (CDR) of the Wa-CRI$^+$ IgM-RF Sie. The antipeptide antibody reacted with the Sie protein, but not with pooled human Ig. A subsequent antiidiotype, produced by immunization with a synthetic peptide corresponding to the second CDR of the IgM-RF Sie L chain, recognized 10 out of 12 human IgM-RF paraproteins (15, 16). The latter results suggested that the majority of IgM-RF L chains are encoded by a single germline V_k gene, or a family of closely related V_k genes (16).

In the present experiments, the CDR structures on RF H and L chains have been probed with seven different peptide-induced antiidiotypic antibodies. A panel of 16 human monoclonal IgM-RF was screened by immunoblotting with the antipeptide antibodies. The results show that the majority of RF share at least two L chain–associated CRI, but not H chain–associated idiotypes. Apparently, antibodies with anti-IgG autoantibody activity repeatedly utilize a common κ L chain sequence, but diverse H chain sequences. Thus, in contrast to the L chains, RF H chains may employ a large number of V_H genes.

Materials and Methods

Preparation of Synthetic Peptides and Antipeptide Antibodies. The seven synthetic peptides listed in Table I (17–19) were prepared by Merrifield's (20) solid-phase method, slightly modified as previously described (14, 15, 20, 21). Each peptide (P) was assigned a four-letter code, designating the corresponding parental protein (S, W, P), heavy or light chain (H, L), and complementarity determining region (1–3). The peptides were conjugated via cysteine to keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl *N*-hydroxysuccinamide ester (22). In initial experiments, rabbits were immunized twice with conjugates emulsified in complete Freund's adjuvant (CFA), then boosted twice with glutaraldehyde-

TABLE I
Amino Acid Sequences of the Synthetic Peptides

Peptide name*	Protein	Residues spanned‡	Amino acid sequence§
PSL2	Sie	49–61	YGASSRATGIPDR(C)
PSL3	Sie	88–99	CQQYGSSPQTFG
PSH2	Sie	49–65	GSPAKWTDPFQGVYIKWE(GGC)
PSH3	Sie	95–102	EWKGQVNVNPFDY(GGC)
PWH2	Wol	49–65	GQIPLRFNGEVKNPGSVV(GGC)
PWH3	Wol	95–102	EYGFDTSDYYYYY(GGC)
PPH2	Pom	49–65	AWKYENGNDKHYADSVNG(GGC)
PPH3	Pom	95–102	DAGPYVSPTFFAH(GGC)

* The first letter, P, designates the peptide, the second letter designates the corresponding parent protein, the third letter designates the heavy or light chain, and the fourth letter designates the CDR.

‡ Numbered according to Kabat et al. (17).

§ The amino acid sequences were reported previously (13, 18, 19). The underlined residues belong to the adjacent framework regions (FR). The residues within the parentheses were added to the C-terminus for coupling purposes. The one-letter amino acid code is used.

crosslinked peptides in incomplete adjuvant, as described previously (14, 15). Thereafter, antisera were obtained and characterized. Subsequently, the immunization procedure was changed to two injections of conjugates in CFA at 1-mo intervals. This scheme generated antibodies with the same specificity as the original protocol.

Preparation of Proteins. Plasma or purified proteins from patients with monoclonal IgM cryoglobulins were generously provided by Drs. J. D. Capra (University of Texas Southwestern Medical School, Dallas, TX) G. Abraham (University of Rochester School of Medicine, Rochester, NY) J. Johnson (Nashville, TN) D. Normansell (University of Virginia, Charlottesville, VA) and H. Metzger (National Institutes of Health, Bethesda, MD). The IgM cryoglobulins were purified by repeated precipitation at 4°C, followed by chromatography on Sephadex G-200 in 0.1 M acetic acid (23), or Ultrogel AcA 22 in 0.2 M sodium acetate, pH 3.5. 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. Bence-Jones κ L chains, prepared from patients' urine by ammonium sulfate precipitation, were the generous gift of Dr. Elliott Osserman (Columbia University College of Physicians and Surgeons, New York). The κ subgroups of the various L chains were kindly determined by Dr. Alan Solomon (University of Tennessee, Knoxville, TN) using subgroup-specific antisera (24).

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA method for idiotype detection has been described previously (14, 15). Briefly, antigens in borate-buffered saline (BBS), 0.1 M borate, 0.2 M NaCl, pH 8.2, were used to coat microtiter plates at 100 μl/well. After blocking with 1% bovine serum albumin (BSA), 100 μl of samples diluted in BBS containing 0.5% BSA were distributed to duplicate wells. The plates were incubated for 3 h at room temperature, and the bound antibodies were quantified by the binding of human IgG–adsorbed, alkaline phosphatase–labeled goat anti–rabbit IgG antibodies (Kirkegaard and Perry, Gaithersburg, MD).

Immunoblotting. The reactivities of antipeptide antibodies with Ig L and H chains were analyzed by the Western blot method (25), with some modifications (14, 16, 26). Briefly, 20 μg of each individual protein (13, 23) or pooled human IgG in gel buffer were loaded onto each slot of a 10% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (27). After electrophoresis (2.5 h at 30 mA), the proteins in the gel were transferred electrophoretically to nitrocellulose papers (2 h at 70 V). Protein-binding sites on the papers were quenched with 0.01 M phosphate-buffered saline, pH 7.2, containing 5% BSA for 1 h at room temperature. Subsequently, the papers were overlaid with the indicated antisera in BBS for 1 h. After washing with BBS, the papers were incubated with ^{125}I-labeled protein A (1 mCi/mg, 2×10^5 cpm/ml) for another hour. Then, papers were washed with BBS, dried, and finally exposed to Kodak XAR-5 film overnight at −70°C.

Results

Expression of Two Major CRI on Human IgM-RF L Chains. All rabbits produced high titers (1:10,000) of antipeptide antibodies, as measured by ELISA (data not shown). Depending upon the immunizing peptide, approximately half of the animals produced antibodies that reacted with the L or H chains of the parent protein, as assessed by immunoblotting. These latter antibodies were used to analyze idiotypic crossreactions among multiple IgM-RF.

Previously, we showed that 10 out of 12 (83%) human monoclonal IgM-RF bear the PSL2-associated CRI marker (16) (Table II). Subsequently, five additional IgM-RF were analyzed for the PSL2-CRI. Fig. 1 *a* (*left*) shows that anti-PSL2 antibodies react with three out of these five new IgM-RF. Together with our previous data, 13 out of 17 (76%) IgM-RF express the PSL2-CRI. This figure is close to the 65% of IgM-RF that express the Wa-CRI (11, 28). The

TABLE II
Idiotype Expression of 17 Human Monoclonal IgM-RF

IgM-RF	PSL2*	PSL3‡	PSH3	PWH2	PWH3	PPH2	PPH3
Cur	++	++	−	−	−	−	−
Gar	++	++	−	−	−	−	−
Glo	++	++	−	−	−	−	−
Got	++	++	−	−	−	−	−
Neu	++	+	−	−	−	−	−
Pal	++	++	−	ND§	ND	ND	ND
Pay	++	++	−	−	−	−	−
Pom	−	−	−	−	−	++	++
Sie	++	++	++	−	−	−	−
Wol	++	−	−	++	++	−	−
Boc	++	++	−	−	−	−	−
Flo	++	++	−	−	−	−	−
Gal	−	++	+	−	−	−	−
Lew	++	−	−	−	−	−	−
She	−	−	−	−	−	−	−
Lay	−	−	−	ND	ND	−	−
Teh	++	ND	−	ND	ND	ND	ND
Total positive	13	11	2	1	1	1	1
Total assayed	17	16	17	14	14	15	15
Percent positive	76	69	12	7	7	7	7

* The results of five RF are in Fig. 1 *a*. The other results are from Chen et al. (16).
‡ Data on first nine RF are from F. Gõni, P. P. Chen, B. Pons-Estel, D. A. Carson, and B. Frangione. 1985. Preferential V_K and J_K utilization in human monoclonal IgM autoantibodies (rheumatoid factors). Manuscript in preparation.
§ ND, not done.

results lend further support to the possibility that the second CDR on RF light chains (L2) is the structural correlate of the Wa-CRI.

It remains controversial whether somatic mutations in Ig variable regions are linked to the Ig class switch (29–31), or whether they occur commonly in IgM molecules (32–34). The IgM-RF L chains, which bear the PSL2-CRI, are likely encoded by a single V_k gene or a family of closely related V_k genes (16). To estimate the extent of diversification of $PSL2^+$ RF L chains, antibodies were generated against the third CDR of the IgM-RF Sie κ chain (PSL3). Immunoblotting with the two antipeptide antibodies enabled us to determine whether the anti-PSL2- and anti-PSL3-defined sequences cosegregated among a panel of IgM-RF proteins from unrelated individuals.

Three rabbits were immunized with the PSL3 peptide conjugated to KLH. One rabbit antisera reacted well with isolated Sie L chains and with the intact IgM-RF Sie protein (Table III and Fig. 2). Antibody binding to both intact Sie, and to Sie L chains was inhibited by the free PSL3 peptide (data not shown).

The reactivity of the anti-PSL3 antibody against a panel of 16 human monoclonal IgM-RF was analyzed by immunoblotting. As shown in Fig. 2, the anti-PSL3 bound to only two out of three known $Wa\text{-}CRI^+$ RF, indicating that the third CDR of the L chain is unlikely to be the structural basis of the Wa-CRI. Fig. 1 *b* (*right*) and Fig. 3 show that anti-PSL3 reacts with three additional RF, but with none of six κ Bence-Jones proteins. Among these six L chains, Eps and

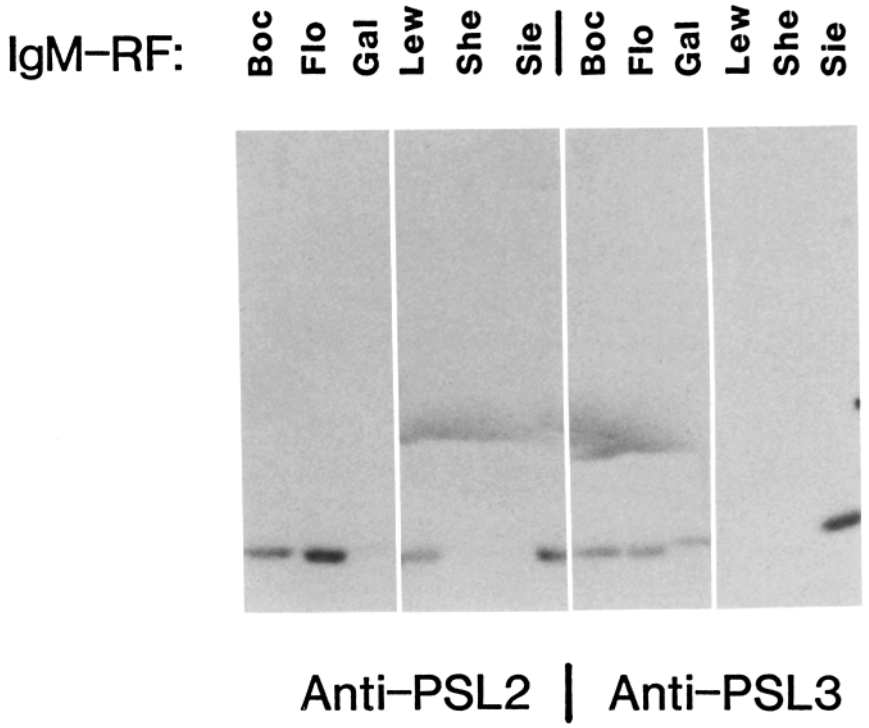

FIGURE 1. Immunoblot analysis of the anti-PSL2 (at 1:500 dilution) and anti-PSL3 (at 1:50 dilution) antisera. About 20 μg of each IgM-RF paraprotein (except Gal 10 μg) was loaded in each lane. After electrophoresis and transfer of samples, the papers were reacted with the indicated antisera, developed with radiolabeled protein A, and finally exposed to XAR film overnight. Protein Gal is the abbreviation of Galv.

TABLE III

ELISA of Anti-PSL3, Anti-PPH2, and Anti-PPH3 Antisera

Exp.	Antigens ($A_{405} \times 10^3$)*			
	Antisera	BSA	Peptide	RF
1	Anti-PSL3	3	836	309
	NRS	7	26	151
2	Anti-PPH2	3	901	66
	Anti-PPH3	10	1,052	181
	NRS	0	1	26
	Buffer	9	15	13

* Antigens were used at 2 μg/ml, and antisera were used at 1:100 dilution.

Wie belong to the κ I subgroup, Mcg belongs to the κ II subgroup, and the rest belong to the κ III subgroup. Thus, the anti-PSL3 defines a primary sequence–dependent CRI that is expressed by 69% of IgM-RF paraproteins (Table II). Furthermore, the PSL2- and PSL3-defined CRI are often found together on the same L chain.

Expression of Three H Chain Idiotypes Associated with Two Wa-CRI$^+$ IgM

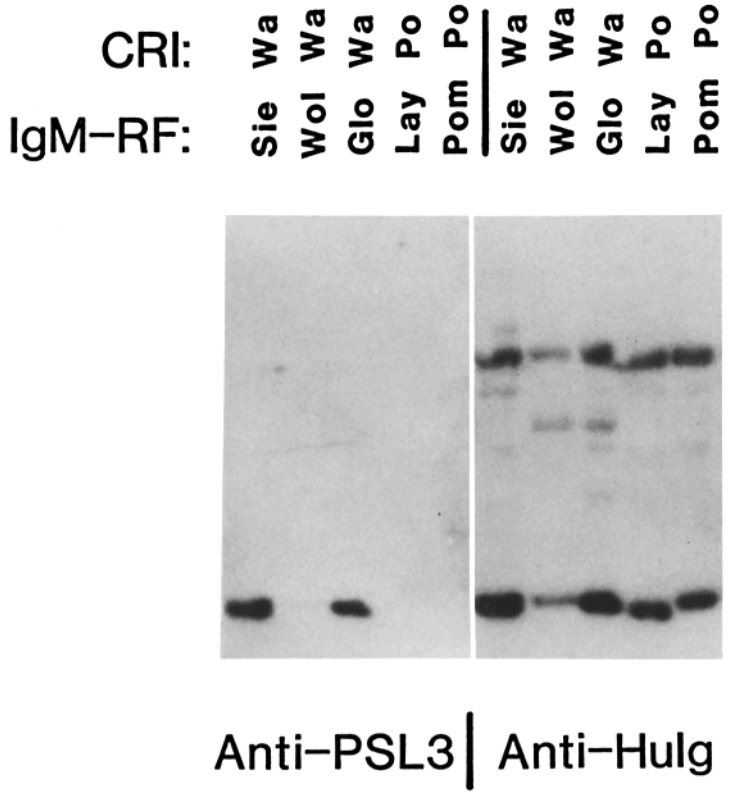

FIGURE 2. Immunoblot analysis of RF bearing either the Wa-CRI or the Po-CRI. The experiment was performed as described in Fig. 1, except that only 8 μg of Wol was loaded, and that polyspecific rabbit anti-human Ig antibodies (IgG fraction, at 5 μg/ml) were used to show the relative amount of polypeptides blotted onto nitrocellulose paper.

RF. Synthetic peptide-induced antibodies and immunoblotting were used similarly to study the idiotypes associated with RF H chains. Four peptides were synthesized, corresponding to the second and third H chain CDR of the IgM-RF Sie and Wol. These two proteins express the dominant Wa-CRI. Antisera from PSH2-immunized rabbits reacted with the free peptide, but not with Sie H chains. In contrast, anti-PSH3 from all four immunized rabbits reacted specifically with the intact IgM-RF Sie, and with isolated Sie H chains (14), Fig. 4 and Fig. 5*c* (*lower left*). 17 different IgM-RF were probed with the PSH3-induced antiidiotype. Only one RF, other than Sie, was weakly positive.

Both the PWH2 and PWH3 peptides induced rabbit antibodies that recognized the isolated H chains of IgM-RF Wol. Fig. 5*d* (*lower right*), and Figs. 6 and 7 show that only Wol is positive for the PWH2- and PWH3-defined idiotypes, among 14 IgM-RF analyzed.

Expression of Two H Chain Idiotypes Associated with Po-CRI$^+$ RF. The Po-CRI antigen is present on 20% of human monoclonal IgM-RF (11). Po-CRI$^+$ IgM-RF have similar V_H sequences (13), particularly in the second and third CDR (H2 and H3). Peptides corresponding to the H2 and H3 regions of the IgM-RF Pom

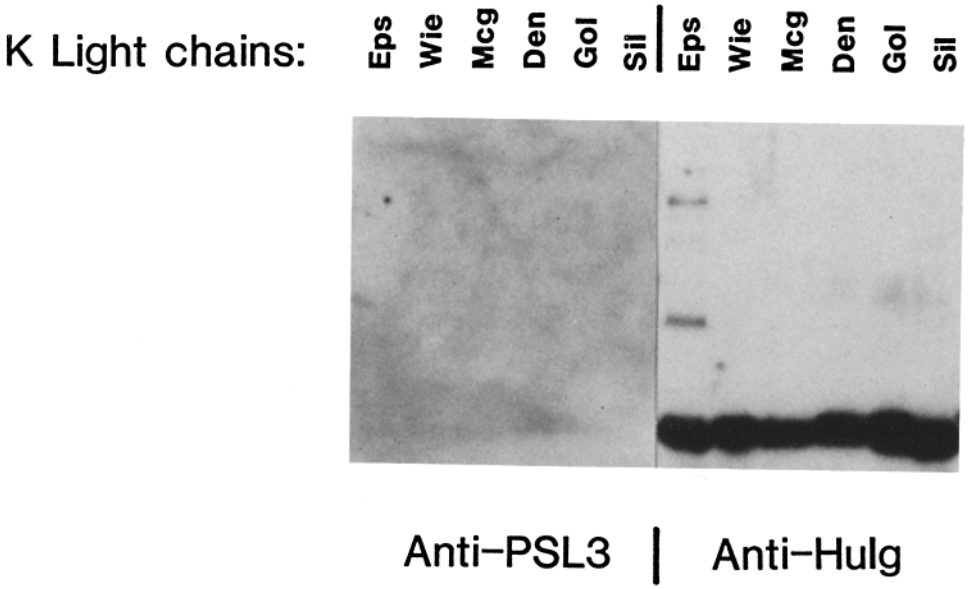

FIGURE 3. Immunoblot analysis of six Bence-Jones κ L chain proteins. The methods are the same as in Fig. 2. Protein Mcg is the abbreviation of McGee.

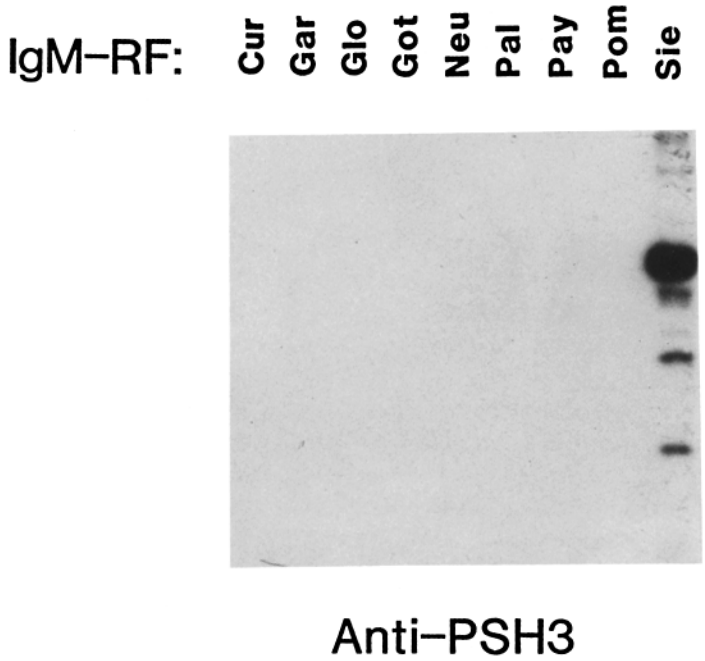

FIGURE 4. Immunoblot analysis of the anti-PSH3 antiserum. The methods are the same as in Fig. 1.

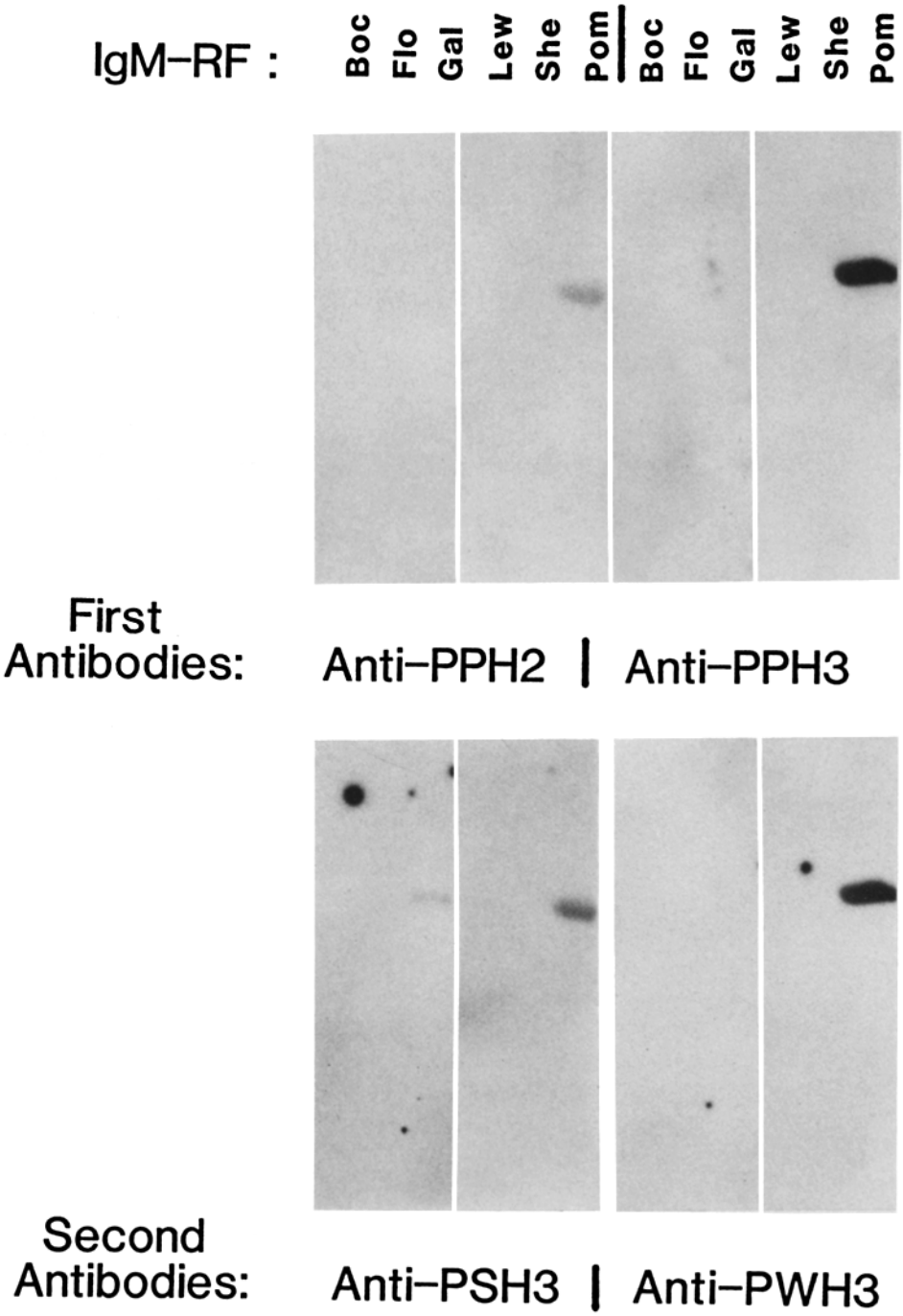

FIGURE 5. Immunoblot analysis of six IgM-RF with four different peptide-induced antiidiotypic antisera. The experiments were performed as in Fig. 1, except that the papers were reacted first with either anti-PPH2 (*a*) or anti-PPH3 (*b*), and then with either anti-PSH3 (*c*) or anti-PWH3 (*d*). This modification was necessitated by the very limited supply of these IgM-RF.

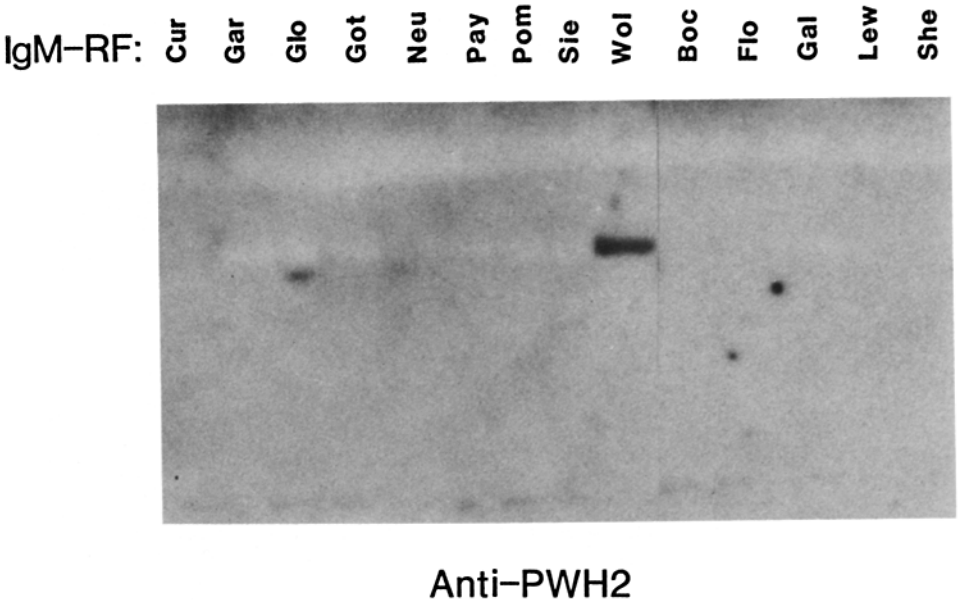

FIGURE 6. Immunoblot analysis of 14 IgM-RF with the anti-PWH2 (at 1:10 dilution) antiserum. The protocol was the same as in Fig. 1, except that phosphate buffered saline was used through the whole experiment.

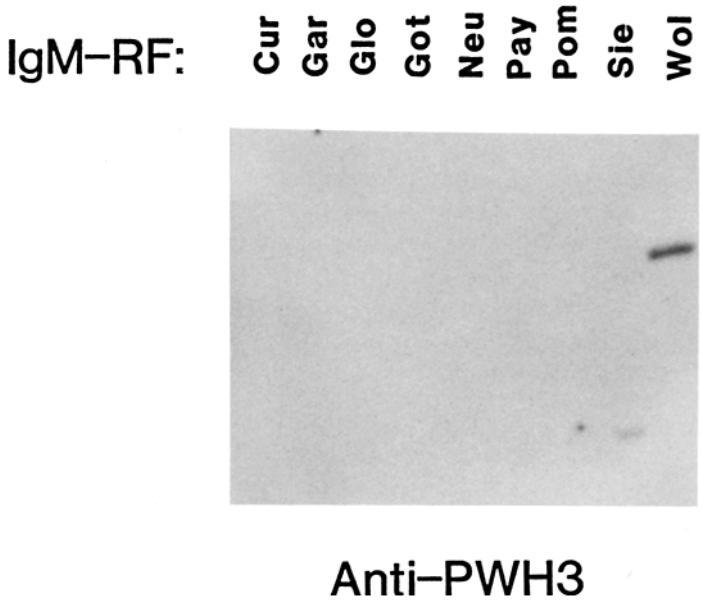

FIGURE 7. Immunoblot analysis of nine IgM-RF with the anti-PWH3 antiserum. The methods are the same as in Fig. 1.

were synthesized (Table I) and used to immunize rabbits. All PPH3-immunized rabbits, and one of two PPH2-immunized rabbits, produced antibodies reactive with the parent protein Pom (Table III). Fig. 5 (*a* and *b*, *top*) and Fig. 8 show

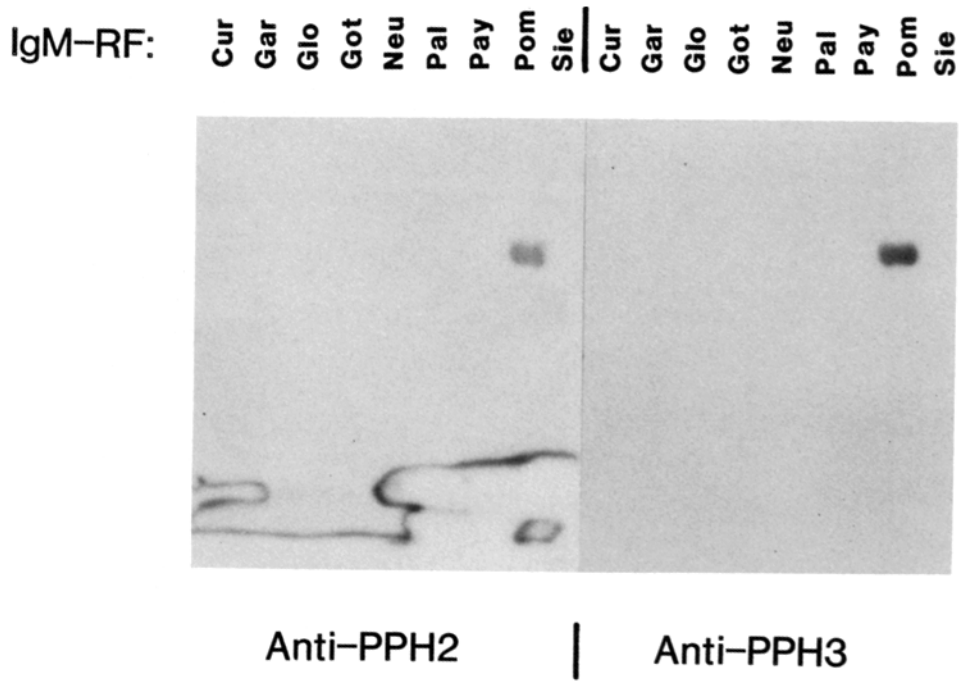

FIGURE 8. Immunoblot analysis of nine IgM-RF with the indicated antisera. The methods are the same as in Fig. 1.

that anti-PPH2 and anti-PPH3 antibodies reacted well with Pom H chains, but not with the other RF analyzed.

Discussion

Synthetic peptides were used to generate seven specific antiidiotypic antibodies against human IgM-RF. These reagents were tested against a large panel of IgM-RF paraproteins. The results demonstrate that the majority of human monoclonal IgM-RF share two primary sequence–dependent CRI, corresponding the second CDR and third CDR of the κ L chain. An analysis of the amino acid sequences of four RF expressing both the PSL2- and PSL3-CRI revealed complete identity in the whole V_k gene region (35). In addition, five RF bearing the PSL2-CRI have almost identical sequences, with only one to four different amino acid residues per RF L chain (12).

In contrast to the recurrent CRI on IgM-RF L chains, the H chain idiotypes are extremely private. Thus, all of four antiidiotypes against synthetic peptides, corresponding to different H chain CDR, reacted only with the parent proteins. A fifth peptide-induced antiidiotype reacted weakly with one additional IgM-RF. These results suggest either that RF H chains are encoded by a number of different V_H and D_H (diversity regions of H chain of Ig) genes, or that IgM-RF H chain sequences reflect an unusually high degree of somatic mutation in a limited number of V_H genes, as well significant variation in VDJ (variable, diversity, joining region) gene joining.

Crossreactive idiotypes represent phenotypic markers for V_H and V_L genes (36,

37). For L chains, the V_L genes encode amino acid residues up to about position 95 (i.e. including ⅔ of CDR3 region) (38, 39). The V_H genes encode amino acid residues up to about position 94, the last amino acid residue of the third framework region. The third CDR of H chains (H3) is encoded by D_H, J_H, and nucleotides of unknown origin (40–42). Only 5–10 D_H genes, and five J_H genes, have been identified in humans and mice. However, imprecise joining at the VD and DJ junctions together with the inclusion of other nucleotides, make the third H chain hypervariable region the most heterogeneous among the six Ig CDR (42–44). This conclusion was demonstrated elegantly by primary sequence analysis of eight murine IgM antigalactan antibodies (45, 46). Thus, it is not surprising that all three antibodies against synthetic peptides, corresponding to the third CDR of RF H chains, recognized private idiotypes.

We anticipated that antibodies against one or both synthetic peptides, representing the second CDR of IgM-RF H chains, would recognize a CRI determinant. In the IgM antigalactan system, the second CDR of the H chain was identical in seven out of eight cases (45). The eight H chains were all encoded by a single V_H gene (34), and four had identical amino acid sequences through the whole variable region. Seven of the IgM antigalactan antibodies also had identical sequences through the whole V_K region (46). By analogy, the fact that the monoclonal IgM-RF H chain–associated idiotypes were unique among 14–15 different RF strongly suggests that the autoantibodies employ a number of different V_H genes.

In conclusion, the synthetic CDR peptide–induced antiidiotypes reveal that 70% of monoclonal human IgM-RF share two light chain–associated CRI. In contradistinction, all five H chain CDR–associated idiotypes were private, among 14–17 monoclonal IgM-RF analyzed. Together, these results establish that the majority of IgM-RF share homologous V_L, but have heterogeneous V_H. It seems likely that one (or very few) V_L genes, but a larger number of V_H genes, are used to encode IgM-RF autoantibodies.

Summary

Recently, we have used synthetic peptides corresponding to the complementarity-determining regions (CDR) of Ig molecules to induce antiidiotypic antisera. Peptide PSH3, representing the third CDR of the IgM rheumatoid factor (RF) Sie heavy (H) chain, induced a private antiidiotype that reacted with only one out of five IgM-RF. Peptide PSL2, corresponding to the second CDR of Sie light (L) chain, induced an antibody against a crossreactive idiotype (CRI), expressed by 10 out of 12 human IgM-RF analyzed. Herein, we report that five additional antiidiotypic antibodies were generated by immunization with synthetic peptides identical to the third L chain CDR of IgM-RF Sie (PSL3), the second and third H chain CDR of IgM-RF Wol, and the second and third CDR of IgM-RF Pom. As analyzed by immunoblot assay, both anti-PSL3 and anti-PSL2 reacted with the majority of 16 IgM-RF. In contrast, all five antiidiotypes induced by the H chain peptides reacted only with the parent proteins, except anti-PSH3, which reacted weakly with one additional RF. These results suggest that one (or very few) V_L gene(s), but a larger number of V_H genes, are used to encode IgM-RF autoantibodies.

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