

## CHARACTERIZATION OF AN EPIBODY

### An Antiidiotypic Antibody That Reacts With Both the Idiotype of Rheumatoid Factors (RF) and the Antigen Recognized by RF

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Since the original discovery of idiotypes (1–3), their structural basis and biological significance have been a central interest in immunology. Recently, Bona et al. (4) described a peculiar antiidiotypic antibody that reacted not only with an idiotype on a monoclonal, human anti-IgG autoantibody (rheumatoid factor, RF),<sup>1</sup> but also with the Fc fragment of human IgG (4). They called this doubly reactive antiidiotypic antibody an epibody, i.e., an antibody recognizing both an idiotope and an epitope on the original antigen.

Very recently, we prepared and characterized three different types of antiidiotypic antibodies against human rheumatoid factors: (a) a monoclonal antiidiotypic antibody (mAb 17.109), that reacts with 30% of human monoclonal RF (5); (b) antiidiotypic antibody that bears the “internal image” of human IgG, and thus binds to most human RF (6); and (c) antiidiotypes induced by synthetic peptides corresponding to distinct, complementarity-determining regions (CDR) on RF heavy and light chains (7, 8). The latter, CDR peptide-induced antibodies identify preselected idiotypes, with amino acid sequences homologous to the immunizing peptides.

In the present study, we describe the induction of an antiidiotypic antibody against human RF with epibody properties. Surprisingly, the epibody was elicited by immunization with a synthetic peptide. The results provide evidence for the possible molecular basis of the epibody phenomenon.

### Materials and Methods

*Preparation of the Synthetic Peptide.* We synthesized a peptide, designated PGL1, that corresponded to amino acid residues 23–34 (first CDR and Cys of the first framework region) in the light chain of the monoclonal IgM-RF Glo, according to the sequence reported by Capra and Kehoe (9). It is composed of Cys-Arg-Ala-Ser-Gln-Ser-Val-Ser-Ser-Ser-Tyr-Leu-Ala.

The synthesis of the control peptide, PSH3 (corresponding to the third CDR of the

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<sup>1</sup> *Abbreviations used in this paper:* BBS, borate-buffered saline; BSA, bovine serum albumin; CDR, complementarity-determining region; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RF, rheumatoid factor.

heavy chain of the IgM-RF Sie) has been described previously (7). It is composed of Glu-Trp-Lys-Gly-Gln-Val-Asn-Val-Asn-Pro-Phe-Asp-Tyr-Gly-Gly-Cys.

**Immunization of Animals.** The synthetic peptide PGL1 was conjugated via its terminal cysteine to keyhole limpet haemocyanin (KLH) with *M*-maleimidobenzoyl *N*-hydroxysuccinamide ester, as described previously (10). Each of three rabbits was injected subcutaneously with 5 mg of the conjugate, emulsified in complete Freund's adjuvant (CFA). The rabbits were boosted twice: 1 mg on day 30 and 2 mg on day 120. The rabbits were bled at various times and the anti-Glo antiidiotype activity was monitored by enzyme-linked immunosorbent assay (ELISA).

**Purification of Proteins.** Plasma or purified proteins from patients with monoclonal IgM cryoglobulins were kindly provided by Drs. J. D. Capra, G. Abraham, F. Goni, B. Fragnone, J. Johnson, and H. Metzger. The IgM cryoglobulins were purified as described previously (8). 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.

**ELISA.** This was done exactly as described previously (7, 8). Briefly, antigens (2 µg/ml) in 0.1 M borate, 0.2 M NaCl, pH 8.2 (BBS) were used to coat microtiter plates (3590; Costar, Data Packaging, Cambridge, MA) at 100 µl/well. After blocking with 1% bovine serum albumin (BSA), 100-µl 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 (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**Protein Blotting.** The reactivity of the anti-PGL1 antibody with immunoglobulin molecules was analyzed by the immunoblot method (11, 12) with some modifications. Briefly, ~10 µg of individual monoclonal IgM-RF proteins (5, 7, 13) or pooled human IgG, in 20 µl sample buffer supplemented with 0.01% 2-mercaptoethanol, was loaded onto each slot of a 10% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (14). After electrophoresis (2.5 h at 30 mA), the proteins in the gel were transferred electrophoretically to nitrocellulose paper (2 h at 70 V). Protein-binding sites on the paper were quenched with 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 5% BSA, for 1 h at room temperature. Subsequently, the paper was overlaid with anti-PGL1 antiserum for 1 h. After washing with BBS, the paper was developed with <sup>125</sup>I-labeled protein A (1 mCi/mg, 2 × 10<sup>5</sup> cpm/ml) for another hour. After extensive washing with BBS, the paper was dried and finally exposed to Kodak XAR-5 film overnight at -70°C.

**Affinity Chromatography.** The globulin fraction of the rabbit antiserum, prepared by precipitation with a 33% saturated ammonium sulfate, was adsorbed sequentially with a BSA-Sepharose 4B column (13.4 mg/ml × 1.5 ml) and a rabbit IgG-Sepharose 4B column (8.3 mg/ml × 1 ml) to remove nonspecific binding material and any possible rabbit RF activity. The adsorbed antibody was then circulated over a human IgG-Sepharose 4B column (9.5 mg/ml × 2 ml). After removal of nonbound material with BBS, the bound antibody was eluted with 0.1 M glycine-hydrochloric acid, pH 2.5, and then was immediately neutralized with Tris base (Sigma Chemical Co.).

## Results

**Induction of Anti-RF Antibody With the PGL1 Peptide.** After receiving two subcutaneous injections of the PGL1 peptide conjugated to KLH, the rabbits were bled, and their sera were analyzed for antipeptide antibody activity by ELISA. All three immunized rabbits produced anti-PGL1 antibody. Two of the antisera reacted significantly with the corresponding intact IgM-RF Glo (Table 1).

Since IgM-RF Glo reacted with rabbit IgG, an immunoblot assay was used to demonstrate the de facto antiidiotype activity of the anti-PGL1 antiserum (7). IgM-RF Glo and the control, pooled human IgG (Cohn fraction II), were

TABLE I  
*Induction of Anti-RF Antibodies with PGL1 Peptide*

Experiment	Line	Samples	Binding to antigens $A_{405} \times 10^5$ :		
			PSH3	PGL1	Glo
1	1	Buffer only	24	14	—
	2	Normal	111	80	—
	3	Immune 2	98	1,468	—
2	4	Normal	—	—	315
	5	Immune 1	—	—	448
	6	Immune 2	—	—	449

The rabbit was immunized with PGL1-KLH in CFA on days 0, 30, and 120, and was bled on day 70 (immune 1) and 135 (immune 2). The antisera were assayed at 1:100 dilution.

fractionated by electrophoresis under reducing conditions, and then transferred onto nitrocellulose paper. After incubation with the antipeptide antibody and final development with  $^{125}\text{I}$ -labeled protein A, autoradiographs were prepared. Fig. 1 shows that the anti-PGL1 antiserum reacts with the kappa light chain of Glo, but not with the heavy chain of Glo, nor with the light chains of pooled human IgG. Thus, the anti-PGL1 displays antiidiotypic activity.

Subsequently, the specificity of the anti-PGL1 antiserum against a panel of human IgM-RF was examined by the immunoblot method. Fig. 2 shows that anti-PGL1 antibody reacts well with IgM-RF proteins Gar, Glo, Got, and Pal, very weakly with Neu, Pay and Sie (data for Sie not shown), and not at all with IgM-RF Lay. Except for IgM-RF Pal, the amino acid sequences of the first CDR of the kappa chains of these IgM-RF have been reported (9, 13, 15, 16) and are shown in Table II. These results suggest that the majority of the polyclonal anti-PGL1 antibodies recognize a determinant associated with Val-Ser-Ser-Ser (residues 28–31). However, the anti-PGL1 antibodies also react with IgM-RF Got, which has Arg instead of Ser at position 29. One possible explanation is that the antigenic determinant for the majority of antiidiotype is Ser-Ser-Ser, that a lesser fraction recognizes Ser-Ser-Tyr, and that Val serves to enhance the binding of both antibodies through its hydrophobicity (see Discussion). Since both Ser and Tyr share the same functional hydroxyl group, the Ser-Ser-Tyr- and Ser-Ser-Ser-specific antibodies might be expected to have partial cross-reactivity.

*Anti-PGL1 Antibody Reacts With Human IgG.* The epibody described by Bona et al. (4) was prepared against human IgM-RF Glo, which contain the PGL1 sequence. As shown in Fig. 1, the PGL1-induced antiidiotype bound to the isolated gamma chains of human IgG. To assess the structural basis for this cross-reaction, two types of experiments were performed. First, the antibody-binding activity to the gamma chains was inhibited partially by the free PGL1 peptide in solution (Fig. 1). Under the same conditions, an unrelated peptide, PSH3 (7), did not detectably inhibit the reaction (data not shown). However, it should be noted that the peptide inhibition of antibody binding to IgG gamma chain was not as significant as to Glo light chain, reaching a maximum of ~70% (as

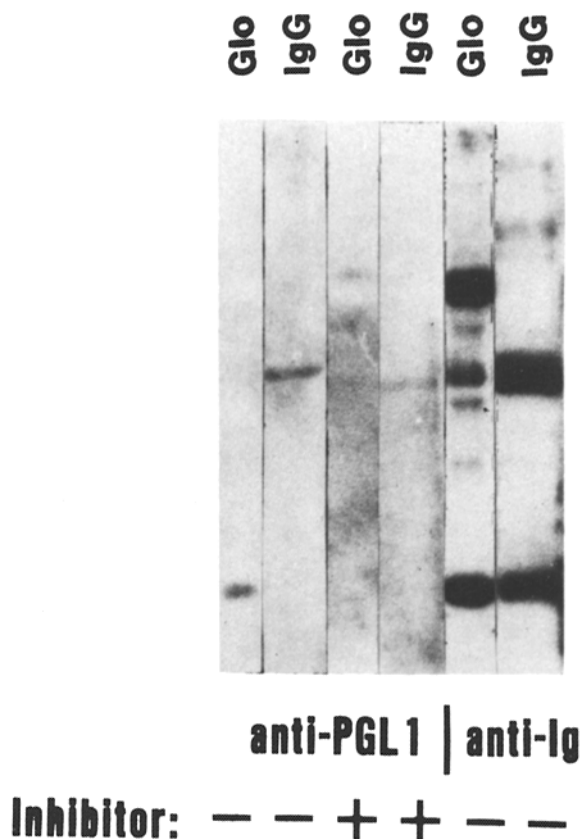


FIGURE 1. The epibody activity of the anti-PGL1 antiserum. 10  $\mu\text{g}$  of each sample was loaded into each slot. The polypeptides were transferred after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were incubated with the indicated antibodies (anti-PGL1 at 1:20 dilution, anti-human Ig at 5  $\mu\text{g}/\text{ml}$ ) and inhibitor PGL1 (50  $\mu\text{g}/\text{ml}$ ).

suggested by the intensity of autoradiographs). This suggests that some anti-gamma antibodies might have been induced nonspecifically or indirectly.

Second, the PGL1-induced epibodies were enriched by affinity chromatography on a human IgG column. As shown in Fig. 3, the eluate from the human IgG column reacted with both the Glo light chain and IgG gamma chains, but not with IgG light chains. However, the binding to Glo light chain, but not to IgG gamma chain, was completely inhibited by the PGL1 peptide. Altogether, these results suggest that a portion of PGL1-induced antiidiotype reacted with intact IgG molecules, and that anti-PGL1 antiserum contains both epibody and anti-gamma antibody that was indirectly induced.

#### Discussion

An antiidiotype with "epibody" properties against the human monoclonal IgM-RF Glo was generated by immunization of a rabbit with a synthetic peptide (PGL1), corresponding to the first CDR of the Glo kappa light chain. The antibody reacted specifically with both the PGL1 and the intact IgM-RF Glo.

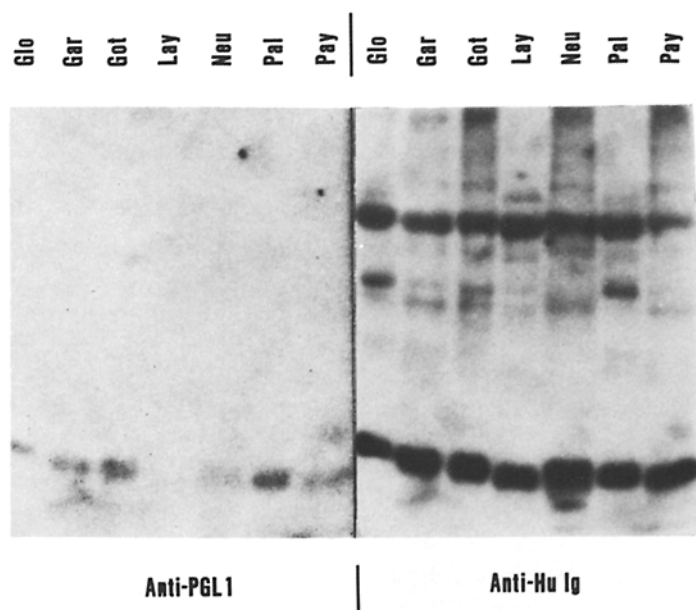


FIGURE 2. The fine specificity of the anti-PGL1 antiserum. Seven human monoclonal IgM-RF were used at  $10 \mu\text{g/slot}$ , and were reacted with the anti-PGL1 antiserum (1:20 dilution) or the anti-human Ig ( $5 \mu\text{g/ml}$ ).

TABLE II  
*Amino Acid Sequences of the Kappa Chains of Human Monoclonal IgM-RF Paraproteins in the First CDR*

IgM-RF	Id	Residue No.												
		24	25	26	27	27A	28	29	30	31	32	33	34	
Glo*	+	Arg	Ala	Ser	Gln	Ser	Val	Ser	Ser	Ser	Tyr	Leu	Ala	
Gar <sup>‡</sup>	+	-----												
Got <sup>‡</sup>	+	-----Arg-----												
Lay <sup>§</sup>	-	Gln	-----					( ) Asn	Val	Asn	Ala	-----Asn		
Neu <sup>‡</sup>	±	-----Arg-----												
Pay <sup>‡</sup>	±	-----Lys-----												
Sie <sup>¶</sup>	±	-----Asn-----												

Residue number according to Kabat et al. (21).

\* Amino acid sequences were reported by Capra and Kehoe (9).

<sup>‡</sup> Amino acid sequences were reported by Ledford et al. (13).

<sup>§</sup> Amino acid sequences were reported by Klapper and Capra (15).

<sup>¶</sup> Amino acid sequences were reported by Andrews and Capra (16).

When analyzed by the immunoblot method, the anti-PGL1 antibody recognized the isolated kappa chains of the IgM-RF paraproteins Glo, Gar, Got, and Pal, but not the kappa chains of IgM-RF Lay. The anti-PGL1 antibody did not bind detectably to the heavy chains of any IgM-RF. The binding of the antiserum to the Glo kappa chain was completely inhibited by the free PGL1 peptide, but not by a control peptide (PSH3). Thus, the synthetic PGL1 peptide induced an antiidiotypic response against a cross-reactive idiotype associated with the kappa chains of

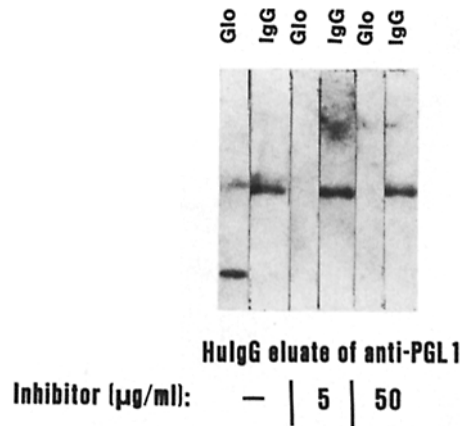


FIGURE 3. The human IgG affinity-isolated, anti-PGL1 antibodies consisted of two sets: the epibody and the anti-IgG antibody. Nitrocellulose paper with polypeptides were incubated with antibodies (0.5  $\mu\text{g}/\text{ml}$ ) and the indicated amount of inhibitor PGL1.

several human IgM-RF autoantibodies. The molecular basis of this cross-reactive idiotype is defined by the PGL1 amino acid sequence.

In our initial observations of the anti-PGL1 antibody, we were surprised and disappointed by its binding with the gamma chain of the pooled human IgG. However, the epibody described by Bona et al. (4) prompted us to study the exact specificities of this peculiar antiserum.

The epibody was defined (4) as an antiidiotype that reacted with the antigen of the idiotype-bearing antibody. Experiments were done to demonstrate that a portion of the PGL1-induced 'polyclonal' antiidiotype did react additionally with human IgG, as a regular antibody reacts with its antigen. Fig. 1 shows that the binding of PGL1-induced antiidiotype with IgG gamma chain was partially inhibited by PGL1, suggesting that there are at least two sets of anti-gamma components in the polyclonal antiserum, and that only one set recognizes the PGL1-associated epitope on the IgG gamma chain. Fig. 3 shows that both sets of anti-gamma antibodies in the serum can be adsorbed by, and eluted from, a human IgG column, demonstrating that they react with intact IgG molecules. In addition, the anti-PGL1 antibodies, previously adsorbed with a human IgG column, still reacted with the Glo light chains (data not shown). In contrast, the human IgG column eluate of anti-PGL1 antisera that was subsequently passed through a PGL1 column reacted only with IgG, and not Glo (data not shown). By pooling these data, the anti-PGL1 antibodies can be classified as follows (Table III): (a) conventional antiidiotype that reacts with Glo light chain only, and whose antibody reactivity is completely inhibited by PGL1; (b) epibody that reacts with both Glo light chain and IgG, and whose antibody reactivity is inhibitable by PGL1; and (c) anti-IgG antibody that reacts with IgG only, and whose binding is not inhibitable by PGL1.

It should be pointed out that the epibody constitutes only a very small portion of the overall anti-PGL1 antibodies, that its binding to the separated Glo light chain and IgG gamma chain is of low affinity, and that the binding to intact Glo

TABLE III  
*Three Components in the Anti-PGL1 Antiserum*

Antibody	Reactivity with:				
	Glo	Glo light	IgG	IgG heavy	PGL1
Anti-Glo, conventional	+	+	-	-	+
Epibody	+	+	+	+	+
Anti-IgG	-	-	+	+	-
	Inhibition of binding by PGL1*				
Anti-Glo, conventional	±‡	+	NA‡	NA	+
Epibody	±	+	-	+	+
Anti-IgG	NA	NA	-	-	NA

\* Inhibition of binding to intact protein was done by ELISA (data not shown).

‡ PGL1 at 500 µg/ml gave about 10% inhibition.

§ Not applicable.

and intact IgG is of even lower affinity. The anti-IgG component was induced indirectly, possibly through the immune network or other unknown mechanisms.

Other than the original description by Bona et al. (4), the existence of epibodies has not been confirmed, so their biological significance could not be investigated. The current paper confirms independently the existence of epibodies. Moreover, it is likely that the structural basis of the epibody described here is the Ser-Ser-Ser sequence shared by the reactive IgM-RF (Glo and Gar) and human IgG molecules [residue 195-197, number according to Kabat et al. (21)]. This is based on the following: (a) Anti-PGL1 recognizes mainly a determinant consisting of Val-Ser-Ser-Ser (Fig. 2); (b) antiidiotypes, induced by a similar peptide PSL1 (which corresponds to the first CDR of Sie light chain and is identical to PGL1 except for having Asn instead of Ser at position 9), did not react with human IgG (unpublished data); (c) anti-PGL1 reacted with the F(ab')<sub>2</sub>, but not Fc, of the human IgG (data not shown); (d) three of six gamma chains with known sequences (Eu, Nie, and IgG G4) have Ser-Ser-Ser at position 195-197, while neither one of two µ chains (Gal and Ou) have Ser-Ser-Ser sequence.

Numerous investigations using dextran and homopolymers of amino acids have concluded that the antibody-combining site can accommodate a hexasaccharide or tetrapeptide (22). In addition, analysis of the antigenic determinant size of the protein antigens revealed that a determinant consisted of a pentapeptide or a tetrapeptide (reviewed in 23). Moreover, in the case of C-terminal pentapeptide of tobacco mosaic (TMV) virus, Leu-Asp-Ala-Thr-Arg, the Leu-Asp could be replaced by *N*-octanoyl (24). This suggested that the anti-TMV antibodies recognized specifically the tripeptide Ala-Thr-Arg, while the Leu-Asp enhanced the antibody binding by providing hydrophobicity. Thus, the loss of idiotype in the RF Pay can be explained by the decreased hydrophobicity, due to the Val → Lys substitution at position 28. Similarly, it is interesting to note that IgG has Val-Pro at the N-terminal to Ser-Ser-Ser (21).

### Summary

Recently, an antiidiotype to human monoclonal IgM anti-IgG autoantibodies (rheumatoid factors) was found to react also with human IgG. This peculiar

antiidiotype was called an 'epibody'. We describe the induction of a similar epibody by immunization with a synthetic peptide (corresponding to one hyper-variable region of the IgM-RF Glo). The results confirm the existence of epibodies, and provide the possible molecular basis of the epibody phenomenon.

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