Clin Exp Immunol 1997; **107**:269–281

Human autoimmune anti-proteinase 3 scFv from a phage display library

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(Accepted for publication 16 September 1996)

SUMMARY

This is the first study describing recombinant human antibody fragments directed to the autoantigen proteinase 3 (PR3) from an immune B cell source. Detection of these autoantibodies has proven valid for the diagnosis and monitoring of Wegener's granulomatosis. The described antibody fragment (scFv) was isolated from a phage display library prepared from the IgG-positive splenic lymphocytes of a patient with systemic autoimmunity. The cloning strategy was designed to maintain the diversity of the antibody variable gene repertoire, and sequencing of several variable genes demonstrated that all major heavy and light chain families were represented. We found an over-representation of particular heavy chain variable domains in splenic lymphocytes which differ from the ones frequently found in peripheral blood lymphocytes. It was possible to obtain specific scFv to PR3 after a single round of selection and the binding could be inhibited by the patients' sera. Although the antibody fragments in the splenic repertoire were found to be highly mutated, it was interesting to find that the selected scFv showed only limited somatic mutation. Furthermore, we could demonstrate that the removal of the mutations had no effect on binding specificity.

Keywords human V gene phage display libraries scFv antibody fragment autoimmunity proteinase 3

INTRODUCTION

Autoimmune diseases count among the major medical problems of today's industrialized societies. However, the origin of autoantibodies is not yet clear. They might arise directly from the repertoire of germ-line variable domain (V) genes, like antibodies, or from 'disease-specific' V genes. Several mechanisms have been proposed, such as polyclonal B cell activation [1], molecular mimicry [2], or a failure to anergize or induce apoptosis of self-reactive B cells [3].

Wegener's granulomatosis (WG) is a disease of uncertain etiology which produces necrotizing granulomas of the upper and lower respiratory tract in association with necrotizing crescentic glomerulonephritis and vasculitis [4]. Autoantibodies against the neutrophil serine protease proteinase 3 (PR3) are the diagnostic marker in the diagnosis of WG and related vasculitides [5–7]. Disease exacerbation is generally preceded by a bacterial upper airway infection combined with the production of anti-neutrophil cytoplasmic autoantibodies (ANCA) [8–10]. It is uncertain which mechanism underlies the induction of the ANCA-related immune response. ANCA may be directly pathogenic by binding to PR3 which is expressed on the cell surface of primed/activated neutrophils [11]. The treatment of choice in active generalized disease is cyclophosphamide [12]. The mechanism of cyclophosphamide-induced immunosuppression is possibly due to direct cytotoxic effect of the drug on immunocompetent lymphocytes, particularly those that have undergone antigenic differentiation and division.

We are interested in the variable (V) genes which encode antibody-binding sites. We hope that information on the V genes encoding human PR3 antibodies and the epitopes recognized may be valuable in elucidating the precise mechanisms underlying the vasculitic process. Studying the human B cell repertoire using hybridoma technology has proved to be very difficult in the case of isolating human MoAbs against self antigens [13,14]. In most cases only low-affinity, cross-reactive IgM antibodies, not representative of pathogenic autoantibodies, were obtained. These limitations have been largely overcome by the display of natural and synthetic antibody V region gene repertoires on the surface of phage [15–18]. Human antibody fragments can be recovered from these libraries against virtually any antigen, including haptens, foreign proteins, cell surface antigen, and self antigen, including human anti-PR3 antibodies [19–22].

To obtain specificities against PR3, we established a V gene

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repertoire derived from the RNA of γ positive splenic B cells from a patient with autoantibodies against PR3 among others. The nucleotide sequence of the V genes of the PR3 antibody was determined and compared with the most homologous germ-line gene in the database. The immunological specificity of the antibody fragment was analysed by ELISA, immunofluorescence, Western blot and inhibition with patient sera. Finally, the role of the mutations of the heavy chain V gene (V_H) on the binding of PR3 was studied by replacing the mutated V_H gene (framework 1, CDR1, framework 2, CDR2 and framework 3) by its germ-line counterpart.

MATERIALS AND METHODS

Patient data

A 55-year-old male with a long history of psoriatic arthropathy was presented with pneumonia and upper airway infection. He had a massive splenomegaly, combined with a severe neutropenia. He had an elevated serum immunoglobulin level of polyclonal nature. The autoantibody screen showed rheumatoid factor activity and autoantibodies against cardiolipin, dsDNA, neutrophil surface antigen and ANCA, the latter being specific for PR3. Neurologically, he had a debilitating peripheral neuropathy, mainly sensory in nature and most likely autoimmune. A vasculitic component was assumed but not proven on histopathological examination. The x-rays showed evidence for interstitial lung disease. The etiology of the progressive lung disease has not been clarified. However, the positive ANCA test was suggestive for an autoimmune component of the lung pathology. There was no evidence for any renal pathology. Before splenectomy, the patient received a Pneumovax vaccination. The splenectomy resulted in a resolution of the neutropenia and the related recurrent upper airway infections. Despite a major improvement in the general condition with a complete normalization of the haematological values and a substantial reduction in immunoglobulin levels, the symptoms of the peripheral polyneuropathy persisted. The autoantibody screen remained positive for rheumatoid factor, cardiolipin and neutrophil antigens. Only the dsDNA antibodies were no longer detectable.

Construction of the IgG-derived V gene phage display library

The spleen tissue was cut into small pieces and squeezed through a sieve. The lymphocytes were isolated by layering the eluate onto a Ficoll gradient of 1·077 g/cm^2. The lymphocytes were harvested from the interface and washed in ice-cold PBS before RNA isolation [23]. In brief, cells were lysed in 5 M guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris–HCl pH 7·5 and 1 mM DTT by vortexing. The RNA was precipitated first with 4 M $LiCl_2$ overnight at 4°C and then with 3 M $LiCl_2$. The isolated RNA was solubilized in 0·1% SDS, 1 mM EDTA, 10 mM Tris–HCl pH 7·5, followed by phenol/chloroform extraction and ethanol precipitation, and stored at –70°C.

The primers used for the amplification of the cDNA are shown in Table 1.

The cDNA synthesis was carried out for the $V_H\gamma$, $V_L\lambda$ and $V_L\kappa$ separately by diluting 4 μg total RNA in 5 μl water. The RNA was added to a 45 μl reaction mixture, resulting in a 50 μl reaction mixture containing 140 mM KCl, 50 mM Tris–HCl pH 8·1, 8 mM $MgCl_2$, 10 mM DTT, 250 mM of each dNTP (dATP, dGTP, dTTP, dCTP), 10 pmol relevant constant region specific primers for IgG, Cκ and Cλ, respectively, and first strand cDNA synthesized. This mixture was heated to 67°C for 5 min before 80 U of human placental RNase inhibitor and 50 U of avian myeloblastosis virus (AMV) reverse transcriptase were added. The mixture was incubated at 42°C for 1 h, heated to 100°C for 3 min, quenched on ice and centrifuged for 5 min.

Polymerase chain reaction (PCR) was used to amplify $V_H\gamma$, $V_L\kappa$ and $V_L\lambda$ genes. Reaction mixtures (50 μl) were prepared containing 5 μl cDNA, 20 pmol of each forward and back primer (equimolar mixture of the family-specific primers), 20 μl dNTP-Mix, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl pH 8·8, 20 mM $MgCl_2$, 100 μg bovine serum albumin (BSA)/ml and 1 U Cetus DNA polymerase. The reaction mixture was cycled 30 times (94°C for 1 min, 60°C for 1 min and 72°C for 1 min). The resulting fragments were gel purified and cycled 25 times (94°C 1 min, 60°C 1 min and 72°C 1·5 min) with flanking primers containing different restriction sites. The cloning into pHenIX (Fig. 1) was carried out in two steps. First, the V_H genes were ligated into pHenIX cut with NcoI and SalI (New England Biolabs, Hitchin, UK) and electroporated into *Escherichia coli* TG1 [24]. Second, DNA containing the V_H library was prepared [23] and each of the fragments containing either the Vκ or Vλ were cloned into pHenIX-V_H cut with Apa L1 and Not1 (New England Biolabs) and electroporated separately into *E. coli* TG1 [24].

Soluble expression of scFv

Single ampicillin-resistant colonies were picked for the production of soluble scFv according to Marks *et al.* [25].

Selection

The phage repertoire was panned using immunotubes (Nunc, Maxisorb, Glasgow, UK) [25,26]. PR3 was coated overnight at 4°C at a concentration of 20 μg/ml in 50 mM carbonate buffer pH 9·6.

ELISA

Single ampicillin-resistant colonies were screened to identify those producing antigen-binding scFv by ELISA essentially as described in Ward *et al.* [27], except that the bound scFv were detected with alkaline phosphatase-conjugated anti-mouse IgG Fc specific (Sigma, Poole, UK). The assay was developed with *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer containing $MgCl_2$ pH 9·7. Reactions were stopped with 50 μl of 3 M NaOH and readings taken at $OD_{405\ nm}$.

Specificity ELISA

The specificity of scFv was determined by ELISA on a panel of antigens: neutrophil extract, myeloperoxidase (MPO), PR3, elastase, lysozyme, lactoferrin, cathepsin G, human serum albumin, BSA, cytochrome C, cardiolipin and H1-stripped chromatin. The ELISA was performed as described.

Competition ELISA

Patient sera were tested for their ability to compete with the scFv fragment for binding to PR3. The ELISA was performed essentially as described above. First, serial dilutions of the scFv were tested in ELISA to determine the scFv concentration corresponding to 75% of the maximal absorbance at 405 nm. This concentration of scFv was mixed with serial dilutions of patient sera before adding them to PR3-coated ELISA plates. Bound scFv were detected as described.

Purification of scFv

The scFv were purified by ion metal affinity chromatography

Table 1. Human V gene primers

Human heavy chain constant region primer																					
IgG1-4 CH1For	5'	GTC	CAC	CTT	GGT	GTT	GCT	GGG	CTT	3'											
Human kappa light chain constant region primer																					
Cκ For	5'	ACA	CTC	TCC	CCT	GTT	GAA	GCT	CTT	3'											
Human lambda light chain constant region primer																					
Cλ For	5'	TGA	ACA	TTC	TGT	AGG	GGC	CAC	TGT	CTT	3'										
Human VH back primers																					
Initial amplification primers																					
VH1back	5'	CAG	GTG	CAG	CTG	GTG	CAG	TCT	GG	3'											
VH2 back	5'	CAG	GTC	AAC	TTA	AGG	GAG	TCT	GG	3'											
VH3 back	5'	GAG	GTG	CAG	CTG	GTG	GAG	TCT	GG	3'											
VH4 back	5'	CAG	GTG	CAG	CTG	CAG	GAG	TCG	GG	3'											
VH5 back	5'	GAG	GTG	CAG	CTG	TTG	CAG	TCT	GC	3'											
VH6 back	5'	CAG	GTA	CAG	CTG	CAG	CAG	TCA	GG	3'											
Reamplification primers with SfiI/NcoI appended restriction sites																					
VH1 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	CAG	GTG	CAG	CTG	GTG	CAG	TCT	GG	3'
VH2 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	CAG	GTC	AAC	TTA	AGG	GAG	TCT	GG	3'
VH3 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	GAG	GTG	CAG	CTG	GTG	GAG	TCT	GG	3'
VH4 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	CAG	GTG	CAG	CTG	CAG	GAG	TCG	GG	3'
VH5 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	GAG	GTG	CAG	CTG	TTG	CAG	TCT	GC	3'
VH6 back SfiI	5'	GTC	CTC	GCA	ACT	GCG	GCC	CAG	CCG	GCC	ATG	GCC	CAG	GTA	CAG	CTG	CAG	CAG	TCA	GG	3'
Human JH Forward primers																					
Initial amplification primers																					
JH1-2 For	5'	TGA	GGA	GAC	GGT	GAC	CAG	GGT	GCC	3'											
JH3 For	5'	TGA	AGA	GAC	GGT	GAC	CAT	TGT	CCC	3'											
JH4-5 For	5'	TGA	GGA	GAC	GGT	GAC	CAG	GGT	TCC	3'											
JH6 For	5'	TGA	GGA	GAC	GGT	GAC	CGT	GGT	CCC	3'											
Reamplification primers with SalI appended restriction sites																					
JH1-2 For SalI	5'	GAG	TCA	TTC	TCG	TGT	CGA	CAC	GGT	GAC	CAG	GGT	GGC	3'							
JH3 For SalI	5'	GAG	TCA	TTC	TCG	TGT	CGA	CAC	GGT	GAC	CAT	TGT	CCC	3'							
JH4-5 For SalI	5'	GAG	TCA	TTC	TCG	TGT	CGA	CAC	GGT	GAC	CAG	GGT	TCC	3'							
JH6 ForSalI	5'	GAG	TCA	TTC	TCG	TGT	CGA	CAC	GGT	GAC	CGT	GGT	CCC	3'							

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Table 1. Continued

Human Vκ back primers

Initial amplification primers

Vκ1 back	5'	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CC	3'
Vκ2 back	5'	GAT	ATT	GTG	ATG	ACT	CAG	TCT	CC	3'
Vκ3 back	5'	GAA	ATT	GTG	TTG	ACG	CAG	TCT	CC	3'
Vκ4 back	5'	GAC	ATC	GTG	ATG	ACC	CAG	TCT	CC	3'
Vκ5 back	5'	GAA	ACG	ACA	CTC	ACG	CAG	TCT	CC	3'
Vκ6 back	5'	GAA	ATT	GTG	CTG	ACT	CAG	TCT	CC	3'

Reamplification primers with ApaL1 appended restriction sites

Vκ1 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CC	3'	
Vκ2 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAT	ATT	GTG	ATG	ACT	CAG	TCT	CC	3'	
Vκ3 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAA	ATT	GTG	TTG	ACG	CAG	TCT	CC	3'	
Vκ4 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAC	ATC	GTG	ATG	ACC	CAG	TCT	CC	3'	
Vκ5 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAA	ACG	ACA	CTC	ACG	CAG	TCT	CC	3'	
Vκ6 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	GAA	ATT	GTG	CTG	ACT	CAG	TCT	CC	3'	

Human Jκ Forward primers

Initial amplification primers

Jκ1 For	5'	ACG	TTT	GAT	TTC	CAC	CTT	GGT	CCC	3'
Jκ2 For	5'	ACG	TTT	GAT	CTC	CAG	CTT	GGT	CCC	3'
Jκ3 For	5'	ACG	TTT	GAT	ATC	CAC	TTT	GGT	CCC	3'
Jκ4 For	5'	ACG	TTT	GAT	CTC	CAC	CTT	GGT	CCC	3'
Jκ5 For	5'	ACG	TTT	AAT	CTC	CAG	TCG	TGT	CCC	3'

Reamplification primers with ApaL1 appended restriction sites

Jκ1 For Not1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACG	TTT	GAT	TTC	CAC	CTT	GGT	CCC	3'
Jκ2 For Not1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACG	TTT	GAT	CTC	CAG	CTT	GGT	CCC	3'
Jκ3 For Not1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACG	TTT	GAT	ATC	CAC	TTT	GGT	CCC	3'
Jκ4 For Not1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACG	TTT	GAT	CTC	CAC	CTT	GGT	CCC	3'
Jκ5 For Not1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACG	TTT	AAT	CTC	CAG	TCG	TGT	CCC	3'

Human Vλ Back primers

Initial amplification primers

Vlλ1 back	5'	CAG	TCT	GTG	TTG	ACG	CAG	CCG	CC	3'
Vλ2 back	5'	CAG	TCT	GCC	CTG	ACT	CAG	CCT	GC	3'
Vλ3 back	5'	TCC	TAT	GTG	CTG	ACT	CAG	CCA	CC	3'
Vλ4 back	5'	TCT	TCT	GAG	CTG	ACT	CAG	GAC	CC	3'
Vλ5 back	5'	CAC	GTT	ATA	CTG	ACT	CAA	CCG	CC	3'
Vλ6 back	5'	CAG	GCT	GTG	CTC	ACT	CAG	CCG	TC	3'
Vλ7 back	5'	AAT	TTT	ATG	CTG	ACT	CAG	CCC	CA	3'

Table 1. Continued

Reamplification primers with ApaL1 appended restriction sites

Vλ1 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	CAG	TCT	GTG	TTG	ACG	CAG	CCG	CC	3'
Vλ2 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	CAG	TCT	GCC	CTG	ACT	CAG	CCT	GC	3'
Vλ3 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	TCC	TAT	GTG	CTG	ACT	CAG	CCA	CC	3'
Vλ4 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	TCT	TCT	GAG	CTG	ACT	CAG	GAC	CC	3'
Vλ5 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	CAC	GTT	ATA	CTG	ACT	CAA	CCG	CC	3'
Vλ6 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	CAG	GCT	GTG	CTC	ACT	CAG	CCG	TC	3'
Vλ7 back ApaL1	5'	TGA	GCA	CAC	AGT	GCA	CTC	AAT	TTT	ATG	CTG	ACT	CAG	CCC	CA	3'

Human Jλ Forward primers

Initial amplification primers

Jλ1 For	5'	ACC	TAG	GAC	GGT	GAC	CTT	GGT	CCC	3'
Jλ2-3 For	5'	ACC	TAG	GAC	GGT	CAG	CTT	GGT	CCC	3'
Jλ4-5 For	5'	ACC	TAA	AAC	GGT	GAG	CTG	GGT	CCC	3'

Reamplification primers with ApaL1 appended restriction sites

JλI For Not 1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACC	TAG	GAC	GGT	GAC	CTT	GGT	CCC	3'
Jλ2-3 For Not 1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACC	TAG	GAC	GGT	CAG	CTT	GGT	CCC	3'
Jλ4-5 For Not 1	5'	GAG	TCA	TTC	TCG	ACT	TGC	GGC	CGC	ACC	TAA	AAC	GGT	GAG	CTG	GGT	CCC	3'

Sequencing primers:

LMB3	5'	CAG	GAA	ACA	GCT	ATG	AC	3'
fdSeq1	5'	GAA	TTT	TCT	GTA	TGA	GG	3'

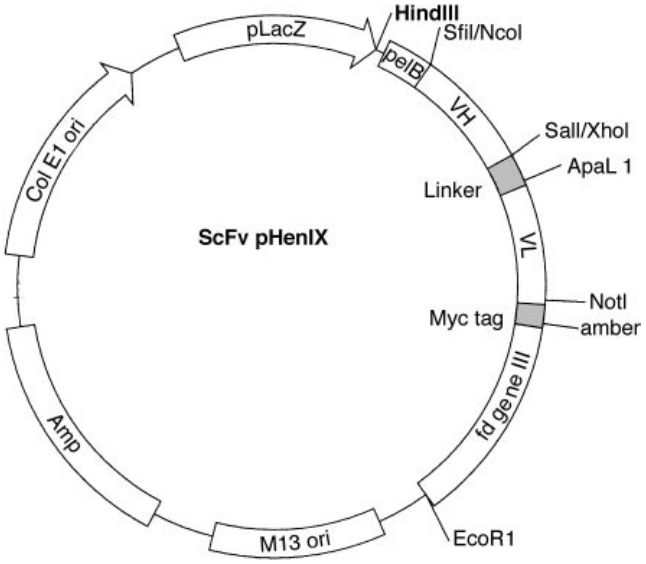

Fig. 1. Structural map of the vector scFv pHenIX. The V_H and V_L genes can be cloned sequentially, V_H as SfiI or NcoI-SalI or XhoI, and the V_L domains ApaL1-NotI.

(IMAC) [28] as described in Griffiths *et al.* [26]. The eluted scFv were further purified by gel filtration and characterized by SDS–PAGE [29].

Western blot

Neutrophil extract (20 μg/ml) was fractionated on a 12% SDS–PAGE and electroblotted onto nitrocellulose. Filters were blocked for 1 h at room temperature in 10% Marvel/PBS. Purified scFv anti-PR3 or serum from the patient of which the library was made, were incubated in Marvel/PBS for 1 h with gentle shaking at room temperature. After washing with PBS–0·05% Tween, binding of scFv was detected by the murine MoAb 9E10, followed by anti-mouse IgG Fc-specific horseradish peroxidase (HRP) conjugate (Sigma) and the human serum by anti-human IgG Fc-specific HRP conjugate (Sigma). HRP was visualized with 3,3′diaminobenzidine tablets (Sigma) in the presence of cobalt ions [23].

Indirect immunofluorescence

The indirect immunofluorescence assay was performed on ethanol-fixed human neutrophils (kindly prepared by Allan Brownlee, Department of Medicine, Addenbrookes Hospital, Cambridge, UK). The scFv anti-PR3 or the germ-line chimaeric construct (0·1 mg/ml) were applied and incubated for 1 h at room temperature. Following three washes with PBS for 5 min, the bound scFv were detected with the MoAb 9E10, followed by anti-mouse IgG Fc FITC conjugate (Dako, High Wycombe, UK). The slides were mounted in citifluor solution and examined by incident light fluorescence microscopy using a Zeiss Axioskop (Zeiss, Jena, Germany). The mouse MoAb 4A3 [30] was used as positive control and an anti-HPA1 and anti-rhesus D scFv as negative controls.

Assessment of insert diversity by BstN1 fingerprinting

The diversity of the library was analysed by BstN1 (New England Biolabs) digestion as described in Clackson *et al.* [31].

Cloning of the scFv anti-PR3 V_H CDR3/V_L into the germ-line VH5 DP73 gene

The scFv anti-PR3 DNA was digested with the restriction enzymes NcoI and Pst1 (New England Biolabs), according to the manufacturer's instructions. The V_H germ-line gene segment DP73 (kindly provided by Ian Tomlinson, MRC Centre for Protein Engineering, Cambridge, UK) was cloned into these sites [23]. The resulting hybrid construct contained the V_H germ-line DP73 gene plus the V_H3 CDR3, the J_H and the complete V_L of the anti-PR3 scFv. The V gene sequence was confirmed by sequencing.

Sequencing of DNA

Sequencing was performed essentially as described by Griffiths *et al.* [26]. Individual clones were PCR-amplified using the primer LMB3 and fdSeq1 (Table 1). PCR cycle sequencing reactions with fluorescent dideoxy chain terminators (Applied Biosystems, La Jolla, CA) were carried out according to the manufacturer's instructions with oligo LMB3 and fdSeq1. Sequencing reactions were analysed on an Applied Biosystems 373A Automated DNA Sequencer and sequence analysis was performed using SeqEd (Applied Biosystems) and Mac Vector 4.5.1 (IBI Kodak, New Haven, CT). V_H genes were compared with germ-line V_H gene segments in the V_H directory compiled by Tomlinson *et al.* [32]. V_L genes were compared with published germ-line Vκ [33] and Vλ [34] gene sequences using the program Seq. Ed (Applied Biosystems). The sequences were compared with the germ-line sequences in the V BASE sequence directory (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK).

RESULTS

Sequence diversity of the IgG spleen library

Following the isolation of the monocluclear cells from the splenic tissue and reverse transcription of the RNA to cDNA, the V_H and V_L genes were PCR-amplified and cloned into the phagemid vector pHenIX for expression as scFv fragments. The V_H genes were cloned first and an IgG V_H library with a diversity of 4×10^6 was obtained. The V_L genes of the λ and κ chains were cloned separately and two libraries were obtained: IgG V_H/λ repertoire was calculated as 8×10^6 and the IgG V_H/κ as 5×10^6. To determine the diversity of the V genes in the IgG library the V genes of 53 $V_H\gamma$, 13 $V_L\lambda$ and 21 $V_L\kappa$ random clones were sequenced and aligned to their most homologous germ-line V gene using the V gene directory compiled by Tomlinson *et al.* (Table 2 and Fig. 2).

Immunoreactivity of the 'non-selected' library

Ninety-six individual bacterial clones from the IgG $V_H/V\lambda$ and the IgG $V_H/V\kappa$, respectively, were induced to produce soluble scFv fragments. The bacterial supernatants were tested on six different antigens (crude neutrophil extract, PR3, MPO, lactoferrin, human serum albumin and chicken egg lysozyme) in an ELISA. There was no detectable reactivity with any of these antigens in the unselected library (data not shown).

Selection on PR3 and characterization of the scFv

The library phage was subjected to three rounds of affinity enrichment on purified PR3. After each round 96 individual bacterial clones were induced to produce soluble scFv fragments which were consequently tested for binding to the selecting antigen in an ELISA. Two clones producing PR3-reactive scFv were

Table 2. V gene usage in the unselected spleen library. Alignment to the most homologous germ-line V genes

Heavy chains

VH1

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DP 3	EVQLVQSGAEVKKPGATVKISCKVSGYTFT	DYYMH	WVQQAPGKGLEWMG	LVDPEDGETIYAEKFQG	RVTITADTSTDTAYMELSSLRSEDTAVYYCAT		
clone 1	q---g----DL----G---F-----AN---	--H--	----------	----IS---V-------	----S--A--G--F----R--------F----	EPNPNYSGLITGKPNAVDD	WGQGT
DP 8	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	GYYMH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG	WVTMTRDTSISTAYMELSRLRSDDTAVYYCAR		
clone 2	--------P--N----------	D--LQ	----------	-------D-S-T-----	R--------LT------T--T----------	AYCSSRTCPFGMDV	WGQGT
clone 3	--------P--N----------	D--LQ	----------	-------D-S-T-----	R--------LT------T--T----------	AYCSSRSCPFGMDV	WGQGT
DP 10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
clone 4	----------Q-----L----------	RN---	----------	-------L---------	--K----------	VGVVDY	WGQGT
clone 5	----------T---S--	N---T	----------	V---M--P-K------A	--S---------T---M---------TF----	GLPFVINDFGDDYYRMDV	WGFGT
clone 6	----------I--	--V--	----------	-------ST--------	-------D----------R---D--------A	QWGIAVIGGDWYFDL	WGRGT
clone 7	----------N	T-T-A	----V--L------	--F-V---PT-------	--------D-T-T----R--T-------F--S	GRSGPRWYHYGLDV	WGQGT
clone 8	----------	HS-L-	-------H--Q-V-	-VV--L-I-K---E---	--------A----V----TG-T-----T------	AVPEGGRYYYGVDV	WGQGT
clone 9	----qe----------	-----	----------	----------	----------S	EPPYSSSGVYYNGMDV	WGQGT
clone 10	-------s----------P--	T----	----------	-S--M---PQ-------	-L----------S----G-T-------F--K	DPVHSGDYFQYS	WGQGT
clone 11	----------N	R---G	----------	------A----------	----N-----R-T--------T----------	LSSVWGSYRFGEYYFDQ	WGQGT
clone 12	----------	----G	----------	-L----A----------	----N-----R-T----G--T----------	LSSVWGSYRFGEYYFDQ	WGQGT
clone 13	----------N	R---G	----------	------A----------	----N-----R-T--------T----------	LSSVWGSYRFGEYYFDQ	WGQGT
clone 14	e----e----------N	R---G	----------	------A----------	----N-----R-T--------T----------	LSSVWGSYRFGEYYLDQ	WGQGT
clone 15	----------R----------	--GV-	----------	Q----L--TH-------	-------K-R--V----------		
clone 16	e----e----------	----N	----------	------D-TT-------	-----SE----------C---	DQDAYARSYYYGMVV	WGQGT
clone 17	----------E-Y-----	-H---	----------	------E-SHH--N---	----S---------L-----T--G--------	GESSWYSDS	WGQGQ
DP 14	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYGIS	WVRQAPGQGLEWMG	WISAYNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR		
clone 18	----------	-----	----------	----------	----------	DVSYCTSTNCYHDAFDI	WGQGT
DP 15	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYDIN	WVRQATGQGLEWMG	WMNPNSGNTGYAQKFQG	RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR		
clone 19	----------	-----	----------	Y---AR----F--R---	----------T----------	LDQRSQYYHGMDV	WGQGT
clone 20	----------	-----	----------	Y---AR----F--R---	----------T----------	LDQRSQYYHGMDV	WGQGT
hv 1263	QVQLVQSGAEVKKPGSSVKVSCKASGGIFS	SYAIS	WVRQAPGQGLEWMG	RIIPILGIANYAQKFQG	RVTITADKSTSTAYMELSSLRSEDTAVYYCAR		
clone 21	e----e------T--------Y-T------	--TV-	----------	----T--VV------R-	---M------T----Q----T----------	GPPYSDIWYDWLDP	WGQGT
clone 22	----------P-----N	----T	----------	----S---S--S--Q---	---F--G-F-N--F----------F--S	SYSENSFYDSFDI	WGQGT
clone 23	----------Q-----L----------	RN---	----------P----	-----Y-VT--------	---V---T----------V-	DGLGITGPDWYFYGLDV	WGQGT
clone 24	----------Q-----L----------	RN---	----------P----	-----Y-VT--------	---V---T----------V-	DGLGITGPDWYFYGLDV	WGQGT
clone 25	--n-re-----Q-----L----------	RN---	----------P----	-----Y-VT--------	---V---T----------V-	DGLGITGPDWYFYGLDV	WGQGT
clone 26	----------Q-----L----------	RN---	----------P----	-----Y-VT--------	---V---T----------V-	DGLGITGPDWYFYGLDV	WGQGT
clone 27	--n-re-----Q-----L----------	RN---	----------P----	-----Y-VT--------	---V---T----------V-	DGLGITGPDWYFYGLDV	WGQGT

VH3

DP 31	EVQLVESGGGLVQPGRSLRLSCAASGFTFD	DYAMH	WVRQAPGKGLEWVS	GISWNSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAK		
clone 28	----------I----K--	-----	-------R--D---	-VN---ND------L--	----------F----------V----R	SRGFNYGSNGFDI	WGQGT
clone 29	----------I----K--	-----	-------R--D---	-VN---ND------L--	----------F----------V----R	SRGFNYGSNGFDI	WGQGT
clone 30	q----q-----GP---------I----K--	-----	-------R--D---	-VN---ND------L--	----------F----------R	SRGFNYGSNGFDI	WGQGT
DP 46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
clone 31	----q--------S----------	N--L-	----T-------VT	---S----------	-------I---------H--GT----M-----	GLLGFCSGGSCYSDY	WGQGT
DP 51	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR		
clone 32	q---q-----P-KA----------	D--VS	----------A	S--G--TS-H-------	----------K----------A--------S-	GTSSITWDGVGK	WGQGT
DP 58	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYEMN	WVRQAPGKGLEWVS	YISSSGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
clone 33	----------V-------	--G--	----------L-	--TRDDYI-EKV--ME-	----------P-----D----------	VDFDL	WGRAS

VH4

DP 65	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	SGGYYWS	WIRQHPGKGLEWIG	YIYYSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
clone 34	----------N----------	-RFL---	----T--R----M-	--DDRETA--------	-I-M-R-A----L--N-T----------	GRGSFTHMLCWNFDL	WGQGT
clone 35	----------V---------I--------R	G-A----	-----P---------	--IP--D---------	L----L----KE---E-R--K----------	AIGDIDRLMAWYFDL	WGRGT

Table 2. Continued

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
clone 36	----------v---------I--------R	G-A----	----P---------	--IP--D---------	L----L----KE---E-R--K----------	AIGDIIDRLMAWYFDL	WGRGT
DP 71	QVQLQESGPGLVKPSETLSLTCTVSGGSIS	SYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
clone 37	----------N-I---A-MR	-D---	----------	-VFH---T------E-	-----M----------TT----------	TAPLS	
clone 38	e---v---------P-----I-----D---	----N	----S---------	----------	----------E----M--L----S-------	GRVYGDYGKFDY	WGQGT
VH5							
DP 73	EVQLVQSGAEVKKPGESLKISCKGSGYSFT	SYWIG	WVRQMPGKGLEWMG	IIYPGDSDIRYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR		
clone 39	q----------EL-S--S	HF--A	----------	---------T-----E-	N---------G-V----------	HRFYYDSSGYFDM	WGQGT
clone 40	q----------NT---T--	T----	---------D---	---------K-------	----------A-----T--Q-----I---V-	HGWQSNWFDA	WGRGT
clone 41	q--v---W----------Y--RSA	NF---	------R-------	----------S--	----------E----------I-----	LSSVWGSFRFGEYYFDL	WGQGT
clone 42	q-n-re----------A------	-----	----------	----------	----------	GLGRSSEYFYYYDMDV	WGHGT
clone 43	-----e-------T----------P	N---A	-----S--------	-------E-------K-	----------S-I-----	GVGRSSDYFYYNYMDV	WGQGT
clone 44	q-n-re----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 45	q----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 46	q----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 47	q---qe----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 48	q----e----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 49	q----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 50	q----------A------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQAT
clone 51	----------	-----	----------	----------	----------	PGLGSSSPQGPLDY	WGQGT
clone 52	-----e----------	-----	----R---------	-----N---------E-	--------PSN----------	PGLGSSSPQGPLDY	WGQGT
VH6							
DP 74	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG	RIYYRSKWYNDYAVSVKS	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR		
clone 53	----e----------I---------	-DT----	----------	----K-Q---E-S-----	---V----------D---E-----	GAGRSLDY	WGQGT

Light chains

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Vλ1 DPL2	QSVLTQPPSASGTPGQRVTISC	SGSSSNIGSNTVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNG	
clone 1	----------A---	--NL-I--T-S-S	--H-----P----V-	NK-----	----------T----------E---	QSQ-SN-S-SGV	FGGGTKLTVG
Vλ1 humlv117	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	ENNKRPS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GTWDSSLSA	
clone 2	-------S---GP---S-A---	T-----T-KS---	----------	G-SN---	-V----------	-A------SWM	FGGGTKLTVLG
Vλ1 DPL5	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	DNNKRPS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GTWDSSLSA	
clone 3	----------	-----D----D--	----------I--	---Q---	----------A--------	---------GV	FGGGTKLTVLG
clone 4	-------S---C--J--------	--G-----S----	----------	-TSQ--P	--------R----------	-S------LYV	FGTGTKLTVLG
clone 5	----------	---N----K-F--	----------V---	--D----	---G----------G----------	G---PT-G-GL	FGAGTKVTVLG
Vλ1 DPL8	QSVLTQPPSVSGAPGQRVTISC	TGSSSNIGAGYDVH	WYQQLPGTAPKLLIY	GNSNRPS	GVPDRFSGSKSGTSASLAITGLQAEDEADYYC	QSYDSSLSG	
clone 6	----------S------	----------	--------S-----S	---I---	-------V-E---L----------	---------SGV	FGGGTKLTVLG
clone 7	nfm----H----S--KT-----	-R--G--ASY----	----R--SS-TTV--	EDYR---	---------IDSS----T-S--KT--------	----G-NQV	FGGGTKLTVLG
Vλ2 DPL11	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	EVSNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYTSSSTL	
clone 8	----------	----------	-------R-------	D-T----	---Y--------D----------T--------	-AHAINRDVL	FGGGTKLTVLG
Vλ2 DPL12	QSALTQPRSVSGSPGQSVTISC	TGTSSDVGGYNYVS	WYQQHPGKAPKLMIY	DVSKRPS	GVPDRFSGSKSGNTASLTISGLQAEDEADYYC	CSYAGSYTF	
clone 9	-------A-A---L--------	------I-H--S--	--------V---I--	--N----	----------V----T------H-	D--T--G--F	FGGGTKLTVLG
Vλ3 DPL16	SSELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNH	
clone 10	----------M----------	-------F---	---------L----	-R------	----------	-------DRHL	FGGGTKLTVLG
clone 11	----------	----------	----------	-------	----------D----------	-------NHLWV	FGGGTKLTVLG
clone 12	----------	----------	-----S---S-V---	-----L-	----------	E------T-LGV	FGGGTKLTVLG
Vλ3 DPL23	SYELTQPPSVSVSPGQTASITC	SGDKLGDKYAC	WYQQKPGQSPVLVIY	QDSKRPS	GIPERFSGSNSGNTATLTISGTQAMDEADYYC	QAWDSSTA	
clone 13	--v--------I S N-	---------G-	----------LM---	---Q---	----------S----------	-----NIVV	FGGGTKVTVLG
Vκ1 DPK1	DIQMTQSPSSLSASVGDRVTITC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP	
clone 1	--v-----P----------	-------I---	----------I---	---I---	-------S------L-----N-----V-----	-E-HT--LS	FGQGTKLEIKR
clone 2	----------	----G------	---H------N----	-V---KV	----------	--LNRFLP	FGQGTRLEIKR
clone 3	--v-----P----------	-------I---	----------I---	---I---	---------S------L---N-----V-----	-E-HT--LS	FGGGTKVEIKR

Table 2. Continued

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Vκ1 DPK3	AIQMTQSPSSLSASVGDRVTITC	RASQGIRNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP	
clone 4	d-v----L-F---------S---	-T--D--E--	-------N-------	S--TVNG	-------A-R-V-E--I---------------	Q-EDLT	FGGGTKLEIKR
Vκ1 A30	DIQMTQSPSSLSASVGDRVTITC	RASQGIRNDLG	WYQQKPGKAPKRLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP	
clone 5	--v----------	--G-P--EYVS	-------R---F--S	E----E-	----------V----S	QTY-RA-RT	FGQGTKVEIKR
Vκ1 L1	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WFQQKPGKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP	
clone 6	--v----------I------I-	-P--DLRTS--	-Y--R-----E----	---R-HT	---A--R-----AF------G----------	L-DHH--IT	FGQGTRLEIKR
Vκ1 DPK5	DIQMTQSPSSVSASVGDRVTITC	RASQGISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	
clone 7	--vK---------L--------	----DL-----	-N----------	-------	----------	---K---YT	FGQGTKVEIKR
Vκ1 L12 (2)	DIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPKLLIY	KASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	
clone 8	--v----------	----T-G----	----R---------H	---T---	----------A----------	----IILYS	FGQGTKLEIKR
clone 9	--v----------	----------	-S---------I---	-T-----	----------	------PYT	FGQGTKVEIKR
clone 10	--v----------	-------G---	----------N----	RT-----	---P--N--A----------A---	------PVT	FGQGTKVEIKR
clone 11	--v----------A----	---LIVGR---	----------S	E--C---	----------A----------SG----	-----LRT	FGQG
Vκ1 DPK9	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	
clone 12	----------L--S-	-T--N---S--	----------R----	---T---	----------A----------S-F-	----TLPYT	FGQGTKLEIKR
clone 13	----------I--L-----	-------T--D	--------S-----H	---NWP-	---A----------E-----I---	--TDNI-VT	FG
clone 14	--v----------	---H--TR---	----------	---T---	--S----------G------V-----	----N--LT	FGGGTKVEIKR
clone 15	--v-----P-----I------V-	----N-TT--H	---L---------F	-----R-	----------DV----L-----	-E-H-P-LT	FGGGTKLEIKR
clone 16	alv----------I--------	----T-D----	----------	G--T-E-	----------H----------	--CSLI-PLT	FGGGTKL
clone 17	--v----------	-P--N--N---	----------N----	---G-HT	----------	--AK-FPYT	FGQGTKVEIKR
Vκ1 DPK10	VIWMTQSPSLLSASTGDRVTISC	RMSQGISSYLA	WYQQKPGKAPELLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSFP	
clone 18	d-v----------K---N-	-V-E---R---	----------	G-----N	EA----------H------R-----Y-----	---QGYLYT	FGQGTKVEIKR
Vκ2 DPK15	DIVMTQSPLSLPVTPGEPASISC	RSSQSLLHSNGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP	
clone 19	e--l----------	------I----DDSLS	--L------R----	-A-----	----------Y----------	--G-E--YT	FGQGTKLEIKR
Vκ3 DPK21	EIVMTQSPATLSVSPGERATLSC	RASQSVSSNLA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWP	
clone 20	---l----------	----N-GI---	--------V---V-S	--Y----	----------D----------	---AE--PELT	FGGGTKLEIKR
Vκ3 DPK22	EIVLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSP	
clone 21	d--m----AS--F----------	-T------N--S	----------S-I-V	A--R---	----------F-	--D-T--YT	LGQGTKLEIKR

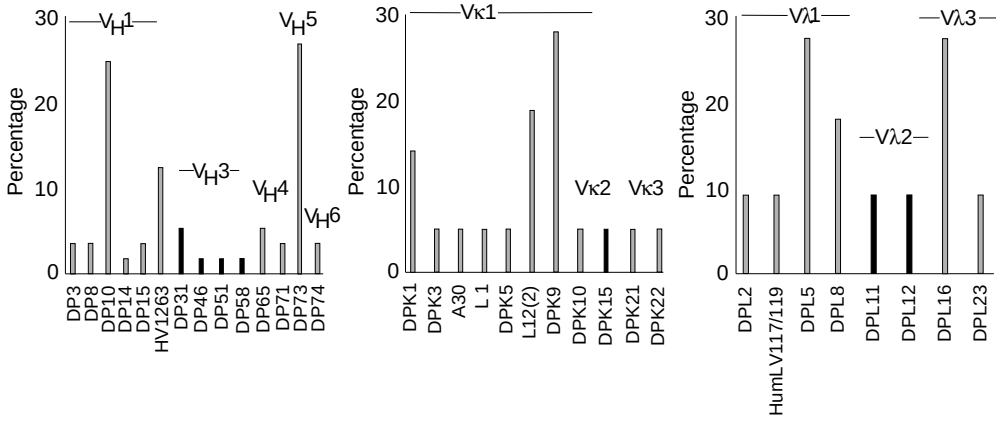

Fig. 2. V gene usage in the unselected immune IgG scFv phage display library (8×10^6)

already observed after a single round of selection, and the number of positive clones increased with each subsequent round. PCR fingerprint analysis of the V gene cassette showed an unique BstN1 digestion pattern. The nucleotide comparison showed that they all derived from the same original clone obtained in the first round (Table 3). The V_H domain was encoded by the DP73 gene from the small V_H5 family with six mutations resulting in five amino acid replacements in the first framework and CDR1. The V_H domain was recombined to a 13 amino acid third hypervariable loop partially encoded by the J_H4 gene. The V_L was derived from the DPκ4 gene of the Vκ1 family and the gene segment had four substitutions, of which two were active, both located in the first framework. The DPκ4 gene was recombined with the Jκ4 gene.

To investigate the importance of the CDR3 domain for the specific binding of the anti-PR3 scFv to PR3, a chimaeric gene was generated. The five amino acid replacements in the V_H domain of the scFv anti-PR3 were removed by replacing the mutated V_H gene segment with the germ-line DP73 gene (Table 3). Investigations by ELISA and immunofluorescence (Fig. 4) convincingly demonstrated that the immunoreactivity of the scFv is not influenced by the five amino acid replacements in the V_H domain.

The specificity of the selected clones was investigated by ELISA using six different protein antigens (Fig. 3). The scFv anti-PR3 reacted specifically with PR3 and the crude neutrophil extract. The specificity was further confirmed by indirect immunofluorescence (Fig. 4), Western blotting (Fig. 5) and competition with patient sera (Fig. 6).

DISCUSSION

The generation of autoantibodies of the IgG isotype against PR3 is a highly specific and sensitive marker for WG, an autoimmune disease characterized by vasculitis with necrotizing granulomas of the upper and lower respiratory tract in association with necrotizing crescentic glomerulonephritis and vasculitis. There are currently no data available on the molecular structure of the V domains of human PR3 antibodies, and studies of the effect of PR3 antibodies on endothelial cells and neutrophil function have been based so far on studies with serum autoantibodies.

We established a patient-derived V gene phage display library using a novel pHen1-derived phagemid vector (pHenIX) allowing the independent cloning of the V_H and V_L gene repertoires. The V gene repertoires were derived from the RNA obtained from the splenic mononuclear cells of a patient with a positive serum screen for PR3, neutrophil-specific membrane autoantigen, dsDNA and cardiolipin, among others. The cloning strategy was designed to maintain the diversity of the repertoire. The sequence diversity of the library was assessed, and demonstrated that all major V_H families were represented combined with a diverse set of V_L genes. Despite the limited number of V_H genes sequenced, some conclusions can be drawn about the V gene usage in splenic lymphocytes. First, some V_H gene segments like DP10 and DP73 seem to be over-represented, while in V_H genes isolated from peripheral blood lymphocytes the segment DP47 is most frequently used [35]. None of the unselected DP73 V_H gene segments is clonally related with the DP73 V_H gene of the scFv anti-PR3. Second, both the V_H and V_L repertoires showed high levels of somatic mutations, the active mutations in the V_H genes being slightly higher than in the V_L genes. The most likely explanation for this is that the V genes are derived from IgG-positive B cells.

The incidence of PR3 reactivity in the unselected library was < 1%. However, after a single round of antigen selection a bacterial

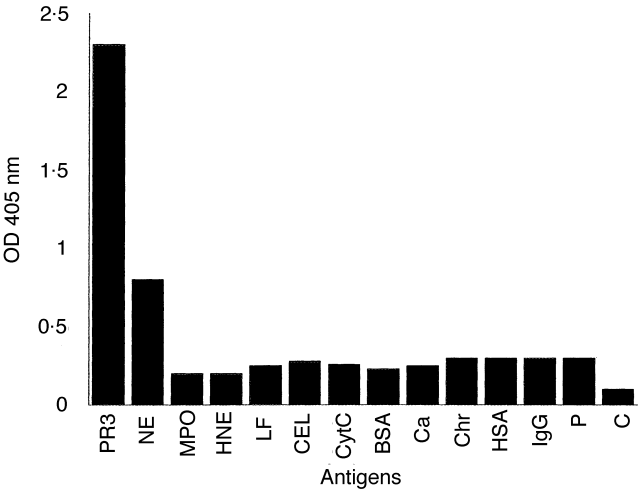

Fig. 3. Specificity ELISA of scFv anti-proteinase 3 (PR3). NE, Neutrophil extract; MPO, myeloperoxidase; HNE, human neutrophil elastase; LF, lactoferrin; CEL, chicken egg lysozyme; CytC, cytochrome C; Ca, cardiolipin; P, plastic; C, control scFv on PR3.

Table 3. Deduced amino acid sequence of scFv anti-proteinase 3 (PR3) and scFv anti-PR3/DP73. Comparison with the DP73 germ-line gene. Dashes indicate identity and replacements in lower case were encoded for the primer

Heavy chain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
V_H5 DP 73	EVQLVQSGAEVKKPGESLKISCKGSGYSFT	SYWIG	WVRQMPGKGLEWMG	IIYPGDSDTRYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR		
scFv PR3	q----e------M---------Q----N--	KH---	----------	----------	----------	LRGQLVRGNYFDY	WGQGT
scFv/DP73	q----------	-----	----------	----------	----------	----------	--

J-gene	Mutations N/AA
J_H4	6/5

Light chain

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Vκ1 DPK4	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QKYNSAP	
scFv PR3	--v------F----------L--	----------	----------	-------	----------	-------LT	FGG

J-gene	Mutations N/AA
Jκ4	4/2

clone was obtained producing a specific monomeric scFv antibody fragment against PR3 which gave the characteristic cytoplasmic immunofluorescence pattern on ethanol-fixed human neutrophils, and in immunoblot the typical 29-kD band was recognized. No reactivity with other antigens was seen by ELISA or immunofluorescence studies. The antibody was not reactive with viable freshly isolated non-activated neutrophils, which is in accordance with results obtained with murine MoAbs against PR3.

The V_H gene DP73 (V_H5) encoding the PR3-reactive scFv had undergone limited somatic mutation, resulting in six nucleotide replacements which resulted in five amino acid replacements. A similar level of somatic mutation was observed in the gene encoding the V_L domain, which had been mutated at four positions which resulted in two amino acid replacements. It is interesting to note that the level of nucleotide replacements in both V genes of the scFv anti-PR3 and of other autoreactive scFv obtained from this library, e.g. U1ARNA [36] and cardiolipin (R. Finnern, PhD thesis) was significantly below the mean level of base substitutions of the random V genes from the unselected library. The reason why the autoreactive V gene segments had undergone limited somatic mutation compared with the non-selected pool of V genes is not answered by our studies, but a possible explanation may be that the self-reactive B cells containing these V genes did not receive appropriate T cell help, or alternatively that clones exposed to further mutational pressure with the inherent possible increase in affinity had been removed from the repertoire.

PR3-reactive recombinant scFv can also be obtained from V gene combinatorial libraries derived from the B cell RNA of normal healthy individuals [22]. The frequency of the PR3 binding clones in the patient-derived library, however, was significantly higher then in the non-immune library. The latter, in which the V_H repertoire was derived from IgM encoding RNA of peripheral blood lymphocytes, had to be subjected to four rounds of phage selection before a binder of low reactivity was obtained, and there was no reactivity in a similar library derived from the IgG repertoire of the healthy individuals. From this we conclude that the frequency of the PR3-reactive binders in the unselected phage population must have been relatively high.

Whether the V_H and V_L gene combination of the anti-PR3 clone resembles the *in vivo* pairing is difficult to answer. However, studies in our group and by others have shown that the recombination freedom of a V_H domain shaped by somatic mutation is limited. If the original V_L domain of such a 'shaped' V_H domain is replaced with an alternative repertoire of V_L domains, either derived from an immune source or from a non-immune source, and subsequently reselected on antigen only the original V_L with minor sequence differences will be selected. This restricted freedom in the use of alternative V_L chain genes, which is probably based on structural restrictions, implies that the V_L gene in the anti-PR3 clone might be at least a 'look-alike' of the original V_L domain.

The binding of the scFv anti-PR3 to PR3 is inhibited by the

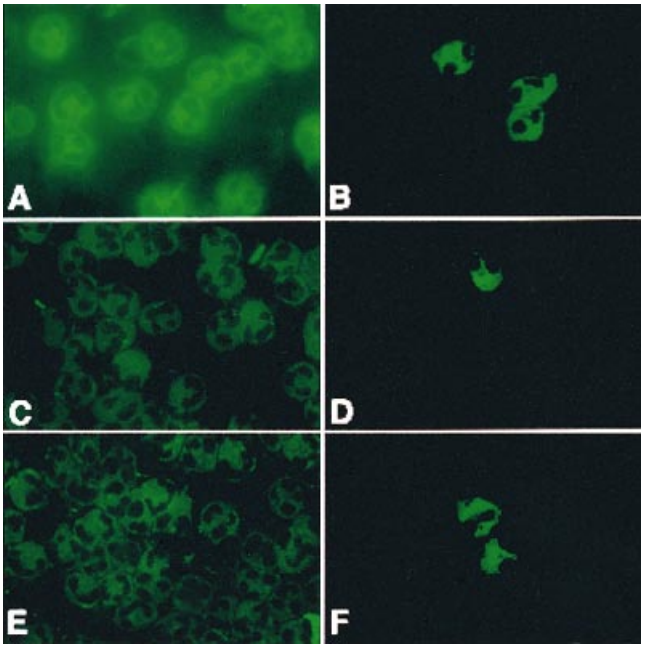

Fig. 4. Immunofluorescence on ethanol-fixed human neutrophils. Left panel, positive immunofluorescence. (A) Anti-proteinase 3 (PR3) MoAb 4A3. (C) scFv anti-PR3 (100 μg/ml). (E) scFv anti-PR3/DP73 (100 μg/ml). Right panel, negative immunofluorescence. (B) Mouse MoAb 9E10 (recognizes the myc-peptide). (D) scFv anti-HPA1 (100 μg/ml). (F) scFv anti-rhesus D (100 μg/ml). The fluorescence seen in the negative controls originates from the staining of eosinophil granulocytes by the anti-mouse MoAb.

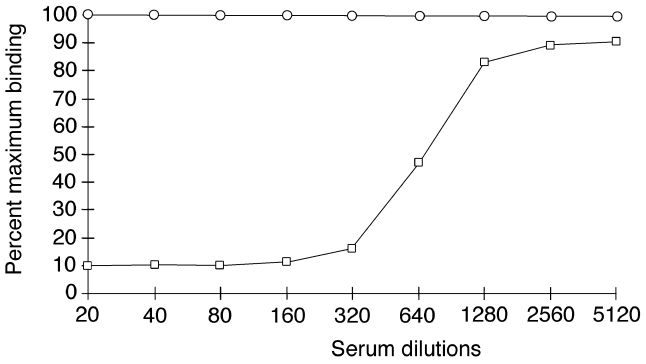

Fig. 6. Competetion of the binding of anti-proteinase 3 (PR3) scFv to solid-phase PR3 by patient serum. ○, Negative serum; □, patient serum.

patient's serum, suggesting that identical or at least overlapping epitopes are recognized.

Results from studies on anti-thyroid peroxidase autoantibodies, which are present in an organ-specific autoimmune thyroid disease [37], show that the antibodies utilize a restricted number of heavy and light chain genes. The V genes were mainly from the V_H1 and the Vκ1 families and the J genes used were from the J_H4, J_H6 and Jκ1 and Jκ4 families. de Wildt *et al.* [36] used the same patient-derived library to select for binding to the autoimmune antigen U1ARNA. They also found one reactive clone. The V_H gene is derived from the V_H4 family DP65 and the V_L is a Vκ1 L12(2).

ACKNOWLEDGMENTS

The scFv anti-HPA1 was a gift from Heather Griffin and the scFv anti-rhesus D

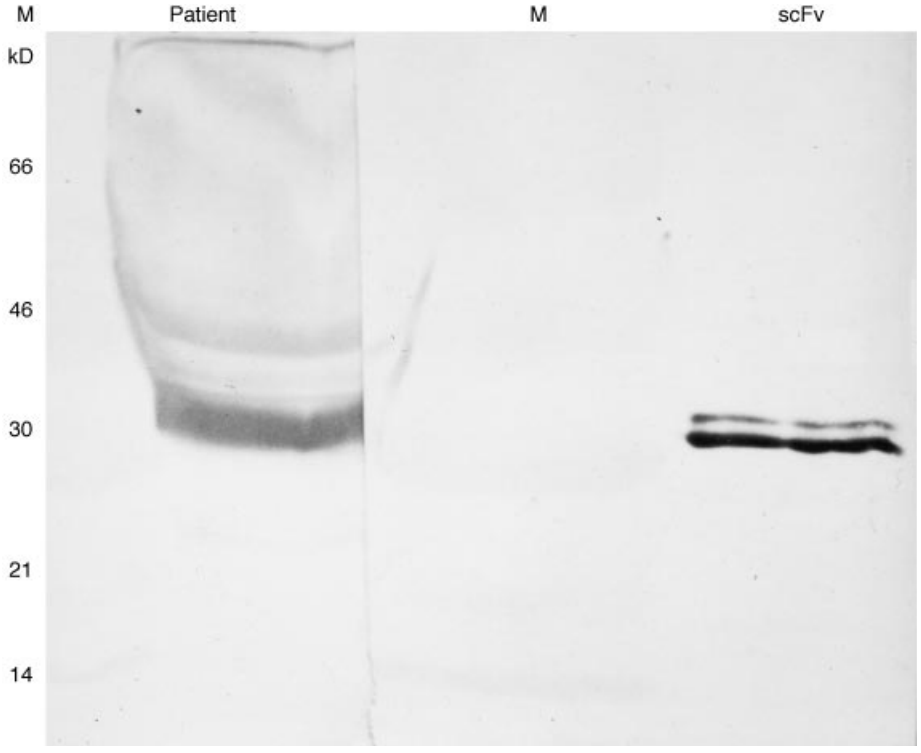

Fig. 5. Western blot on neutrophil extract fractionated on SDS–PAGE. Lanes 1 and 3 (M), molecular weight markers; lane 2, patient serum; lane 4, scFv anti-proteinase 3.

a gift from Eric Timmers. R.F. was supported by the National Kidney Research Fund.

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