A novel system to test for specificity of B cell receptors from tissue of Wegener's granulomatosis patients

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This work was supported by grants from the German Research Society (DFG): to Dr. Mueller from the Clinical Research Unit 170 and from the University of Saarland, Faculty of Medicine, HOMFOR to Dr. Voswinkel.

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Received on March 6, 2008; accepted in revised form on April 1, 2008.

Clin Exp Rheumatol 2008; 26 (Suppl. 49): S92-S97.

Key words: Vasculitis, Wegener's granulomatosis, B lymphocyte immunology, immunoglobulins, B cell receptors, recombinant antibodies, fab.

Competing interests: none declared.

ABSTRACT

Objective. *Wegener's granulomatosis (WG) is characterized by granulomatous inflammation of the respiratory tract and Anti Neutrophil Cytoplasmic Antibody (ANCA)-associated systemic vasculitis. The pathognomonic ANCA in WG is typically directed against proteinase 3 (PR3). Germinal centre-like clusters of lymphocytes were seen in granulomata of WG patients suggesting an antigen-driven maturation of B lymphocytes potentially leading to ANCA formation. The goal of this study was to develop a system to determine the specificity of B cells found in WG granulomata via the generation of fab fragments as antibody analogues. These fab fragments have the identical antigen binding site like the B-cell receptor from which the DNA was derived.*

Methods. *Single B cells were isolated from B cell clusters within the granuloma of a WG patient by laser-assisted microdissection. Their immunoglobulin genes (VH/Vκ, VH/Vλ) were characterized by seminested single cell PCR and cloned into a phagemid vector in order to produce fab fragments. The fabs were characterized by protein gel electrophoresis and western blot.*

Results. *The immunoglobulin genes from lymphocyte infiltrates of WG granulomata reveal antigen-driven selection. On the basis of two individual couples of mutated VH/Vλ PCR products functional fabs were generated that represent the B cell receptors of WG tissue-derived single B cells.*

Conclusion. *This is the first* in vitro *model to test for specificity of B cell receptors from WG granulomata. With respect to ANCA origin in WG this system provides a tool to elucidate the structure-function relationship of apparently antigen-driven maturation of B cells within Wegener's granuloma.*

Introduction

Wegener's granulomatosis (WG) starts as a granulomatous disease out of which the systemic vasculitis develops (1, 2). After the initial granulomatous manifestation in the respiratory tract facultatively systemic vasculitis arises following a variable span of time (3, 4). In more than 90% of WG patients the systemic necrotizing small vessel vasculitis is linked to the detection of Anti-Neutrophil-Cytoplasmic-Antibodies (ANCA) directed against Wegener's autoantigen Proteinase 3 (PR3) (3). *In vitro* and *in vivo* data provide evidence, that ANCA are pathogenetic for systemic vasculitis (5-8). There is a growing number of reports about successful treatment of refractory WG by depletion of B lymphocytes with the anti-CD20 antibody rituximab (9-11).

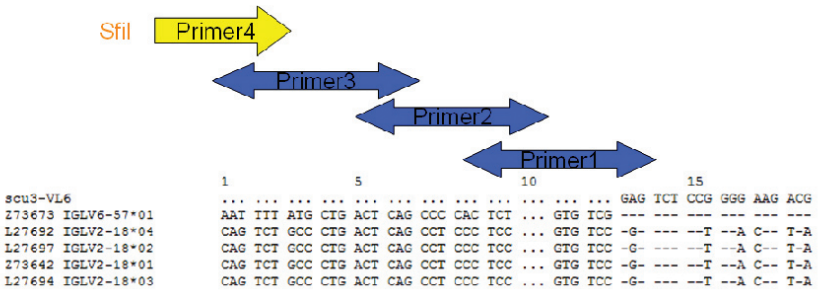
From previous studies we concluded that the granulomatous lesions represent ectopic lymphoid structures which may serve as a breeding-ground where B cells which produce PR3-ANCAs can arise. B cell clusters were found in vicinity to their potential target antigen PR3 as well as plasma cells and follicular dendritic cells within granulomatous formations of endonasal biopsy specimen of WG patients. The analysis of the B cell receptor coding regions revealed antigen-conducted mutation and maturation with clonal expansion like in a germinal centre-like reaction (12-14). Thus, antigen-selected B cell clusters within granulomatous lesions could present the "missing link" between localized, granulomatous and systemic, ANCA-associated disease. Interestingly, our previous studies of B cell maturation in WG demonstrated a skewing of mutations towards negatively charged amino acids, consistent with a potential maturation process on the positively charged PR3 (12).

The goal of this study is to devise a system which enables us to test this hypothesis. To achieve this goal we isolated single B cells from the aforementioned granulomatous lesions. DNA was isolated to produce its resulting antibody recombinantly. There are several approaches for the expression of full-length immunoglobulin molecules that require eukaryotic cellular systems (15). However, an easier way for the expression of immunoglobulin fragments with lower molecular weight can be performed in prokaryotic cells, like *Escherichia coli*. Key to the success of this approach is the phenomenon of the expression of functional antibody fragments by secretion into the periplasm of *Escherichia coli* (16,17). For this purpose fab fragments are a versatile format. Fab fragments are antibody fragments consisting of the light-chain and a fragment of the heavy-chain, its variable region and CH1 domain, respectively. Therefore fab fragments preserve the highly variable antigen-binding part of the antibody. DNA-segments coding for variable heavy- and light-chain of isolated B cells were cloned into the vector pCES-1 (18). pCES-1 represents a phagemid vector allowing expression of fab fragments displayed on infectious filamentous phage particles or as soluble recombinant protein. These fab fragments can consecutively be used to determine the specificities of immunoglobulins expressed by B cell of granulomatous lesions in WG.

Materials and methods

Endonasal biopsy specimens were obtained from a WG patient with granulomatous endonasal manifestation. The endonasal biopsy specimen was immunostained with anti-CD20 to identify B cells. Individual B cells from these granulomata which were located in the aforementioned germinal centre like structures were isolated by laser-assisted microdissection (19). Consecutively, the isolated single cells were subjected to two rounds of VH-, Vκ- and Vλ-specific seminested PCR (20, 21). The resulting couples of VH/Vκ and VH/Vλ, respectively representing B cell receptor encoding genes were directly sequenced using the same oligonucleotide primers

Table I. The individual oligonucleotide primers and conditions for extension PCR.



Scu68vh
Theoretical 5'-primer
GGCCCAGCCGGCCATGGCC CAG GTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAG () ACC CTG TCC CTC ACC TGC GCT G

5'-first GGA CTG GTG AAG CCT TCG GAG () ACC CTG TCC CTC ACC TGC GCT G (74°C)

5'-second CAG CTG CAG GAG TCG GGC CCA () GGA CTG GTG AAG CCT TCG GAG AC (74°C)

5'-third CAG CCG GCC ATG GCC CAG GTG ()CAG CTG CAG GAG TCG GGC CCA (72°C)

5'-fourth GGG GGG GGCC () CAG CCG GCC ATG GCC CAG GTG (74°C)

3'-primer GGG GGG () GGTGACCCCTGTGACCAGGGGTCCTTGGCCC (72°C)

Scu68v λ
5' GGG GGG GTGCAC () cCT TCT GAG CTG ACT CAG GAC CC (72°C)

3'GGG GGG () CTC GAG () GACGGTCAGCTTGGTGCCTCCG (72°C)

Pr3pos VH
5'-first CAG GTG CAG CTG GTG GAG TCT () GGG GGA GGC GTG GTC CAG CC (72°C)

5'-second gcG GCC CAG CCG GCC ATG GCC () CAG GTG CAG CTG GTG GAG TCT G (72°C)

5'-third - GGG GGG () gcG GCC CAG CCG GCC ATG GC (74°C)

3'-GGG GGG GAGGAGACGGTGACCAGGGTTC (74°C)

Pr3pos-V λ
5' -GGG GGG G GTG CA () CAG TCT GCC CTG ACT CAG CCT G (72°C)

3' -Ggg ggg CTCGAGGACGGTCAGCTTGGTCCCTCCG

For each of the DNA segments 5'primers were devised as shown in this table except for Scu68vl. The same 3' primer was used in each round of PCR. The "theoretical primer" is the complete primer sequence after adding all the required nucleotides in one step. Parenthesis () are used to indicate the division between the original sequence and added nucleotides. Six G's were entered to protect the DNA segment from random nuclease activity which could lead to nucleotide loss. Annealing temperature is shown at the end of each primer.
We used Proofstart™ DNA polymerase (Quiagen, Hilden, Germany) for our PCRs and set up the following reaction mixture.

			Final concentration
10x Proofstart PCR Buffer	5	µl	1x
dNTP Mix (10 mM of each)	1.5	µl	300 µM of each
Primer 1	0.5	µl	1 µM
Primer 2	0.5	µl	1 µM
Proofstart DNA Polymerase	1	µl	=2.5 units
Distilled water	40.5	µl	
Template DNA	1	µl	

The following PCR program is used:

Step 1	Activation:	5 min 95°C
Step 2	Denaturation:	1 min 94°C
Step 3	Annealing:	1 min 50-x°C (indicated in parenthesis)
Step 3	Extension	1 min 72°C
Step 4	Repeat	goto step 2 30 times
Step 6	72°C 7 minutes	
Step 7	hold at 4°C forever	

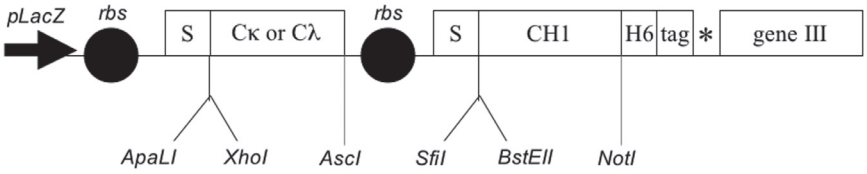


Fig. 1. Phagemid vector pCES1 for display of antibody Fab fragments.
Schematic representation of pCES-1. The polylinker region comprises two signal sequences ("S"; elB and the geneIII leader sequence), the C_κ or C_λ domain, ribosome binding site (rbs), CH1 domain, hexa histidine tag (H6) and a c-*myc* derived sequence (tag). Variable domain genes can be cloned as *Apa*LI - *Xho*I fragments and *Sfi*I / *Pst*I - *Bst*EII fragments. The amber stop codon (*) between the antibody genes and bacteriophage gene III enables the production of soluble Fab fragments in a non-suppressor strain of *E. coli*. Expression of the bicistronic operon is under control of the LacZ promotor (p*LacZ*).

as for the second round of PCR. In our structural analysis functionality as well as mutational patterns were determined (unpublished data). Scu68 is a VH/Vλ couple from a WG patient's single B cell. For positive control we developed a fab based on the sequence of WGH1, an anti PR3 IgMλ antibody that had been characterized by Davis *et al*. (22). To produce a fab based on this sequence we used DNA from a WGH1-producing hybridoma. We ran a PCR on the cell culture material using primers which attach to the sequences and which add the restriction enzyme recognition sites used to clone the DNA which codes for the heavy and light variable domains (Table I).

Table II. Nucleotide and amino acid sequences of the VH and Vλ insertations for fab-generation: Alignement to the respective germline gene.

Scu68VH

```
                <---------------------------------------------- FR1 - IMGT
                1              5              10             15
scu68VH         cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
M29811 IGHV4-61*01 ---- ---- ---- ---- ---- ---- ---- ---- ---- ... ---- ---- ---- ---- ----
                ----------------------------------------------> _____________
                              20             25             30
scu68VH         tcg gag acc ctg tcc ctc acc tgc gct gtc tct gga gtc tcc gtc
M29811 IGHV4-61*01 ---- ---- ---- ---- ---- ---- ---- ---- a--- --- --- --- t -g- --- ----
                __ CDR1 - IMGT _____________<-------------------------
                              35             40             45
scu68VH         gag gat gat act tat tat ... ... tgg acc tgg att cgc cag tcc
M29811 IGHV4-61*01 agc ag- -g- -g- --c --c ... ... --- -g- --- --c --g --- c--
                FR2 - IMGT ----------------------------> _______ CDR2
                              50             55             60
scu68VH         cca ggg aag gga ctg gag tgg att ggc aat gtc tat tac agc ggg
M29811 IGHV4-61*01 --- --- --- --- --- --- --- --- --- ---g t-- a-- --- --- --t ----
                - IMGT _______ <-----------------------------------------
                              65             70             75
scu68VH         aca aac ... ... ... tac tac aac ccc tcc ctc gag ... agt cga
M29811 IGHV4-61*01 -gc  -c- ... ... ... a-- --- ---- ---- --- ---- a--  ... ---  ---
                ---------------------------------- FR3 - IMGT ----------------
                              80             85             90
scu68VH         gtc acc gtt tcg ctg gac acg tcc aac aat cag ttc tct ctg aaa
M29811 IGHV4-61*01 ---- ---- a-a --a g-a ---- ---- ---  --g --c --- --- --c --- --g
                ------------------------------------------------------------> ____
                              95             100            104
scu68VH         ctg agg tct gtg acc tct gcg gac acg gcc ata tat ttt tgt gtg
M29811 IGHV4-61*01 ---- --c --- --- ---- g-- --- ---- ---- --- g-g -- -ac --- -c-
                _____________ CDR3 - IMGT _____________________
scu68VH         aga gat gct tat tat gga ctg ggg tct ccc tca aac tgg ttt gac
M29811 IGHV4-61*01 ---- --

scu68VH         ccc tgg ggc caa gga ccc ctg gtc aca ggg gtc acc gtc tca agc
```

Q V Q L Q E S G P G L V K P S E T L S L T C A V S G V S V E D D T Y Y W T W I R Q S P G K G L E W I G N V Y Y S G T N Y Y N P S L E S R V T V S L D T S N N Q F S L K L R S V T S A D T A I Y F C V R D A Y Y G L G S P S N W F D P W G Q G P L V T G V T V S S

Table II continues on next page

Cloning of variable chain coding DNA-segments into the pCES-1 vector

For expression of fab fragments, the vector pCES-1 was used in a procedure as described before (18, 23). This vector carries all important features for cloning human antibody fragments. Figure 1 shows the cloning schedule and the vector map; the vector contains human C_κ and CH1 (gamma-1) or C_λ and CH1 (gamma-1) genes, and is set up for the expression of fab fragments. For cloning of V_L a *Xho*I restriction site was introduced into the C_λ gene by mutation of CTCGGT to CTCGAG. After successful ligation of V_L the *Xho*I restriction site was again mutated to CTCGGT, since that mutation would code for Glu instead of Gly. Variable domain genes can be cloned as *Apa*LI - *Xho*I fragments and *Sfi*I/*Pst*I-*Bst*EII fragments. Besides the *myc*-derived tag, an additional stretch of 6 histidines is at the 3' end of the *Not*I cloning site, to provide an affinity handle for purification of antibody fragments. The amber stop codon (*) between the antibody genes and bacteriophage gene III enables the production of soluble fab fragments in a non-suppressor strain of *E. coli*. Expression of the bicistronic operon is under control of the LacZ promotor (p*LacZ*). To clone the VH/Vλ single cell PCR products into the pCES vector we performed extension PCRs using the primers and conditions specified in Table I. To facilitate the procedure we successfully run one PCR with a mix of all primers. A maximum of 5 different primers were present in the PCR mix (four 5' primer and one 3' primer).

Expression and purification of soluble recombinant fab antibodies

Fab antibodies were expressed and purified as described previously (15). Cells of Escherichia coli strain TG1 were grown to $A_{600\ nm}$ = 0.8-1.0 and induced to express the recombinant fab antibody by the addition of 1 mM IPTG for 3-4 h at 30°C. The bacterial periplasmic fraction was collected according to the method of Minsky *et al*. (24): briefly, bacterial pellets were resuspended in spheroplasting buffer (100 mM Tris-HCl/0.5 M sucrose/0.5 mM EDTA pH 8.0), containing EDTA-free

Proteinase-Inhibitor Cocktail (Roche, Mannheim, Germany) and the cells were osmotically shocked by the addition of 0.3 × spheroplasting buffer. Cellular debris was pelleted by centrifugation and the supernatant (periplasmic fraction) collected, dialysed (overnight, 4°C) against PBS adjusted to pH8 and applied onto a prewashed Talon column (Clontech, Palo Alto, CA). Bound Fabs were eluted using 0.5 ml of 100 mM imidazole in PBS. The eluted fabs were dialyzed against PBS (overnight, 4°C) to remove residual imidazole.

Western Blot analysis

Soluble recombinant fab fragments were separated by 12% SDS-PAGE and stained with Coomassie (Biorad, Hercules, CA, USA) or transferred to a nitrocellulose membrane (Whatman, Maidstone, UK). Membranes were stained with the indicated primary antibodies: rabbit anti-human-lambda IgG (Dako, Glostrup, Denmark), mouse anti-Penta-His IgG (Quiagen, Hilden, Germany), goat anti-rabbit IgG-HRP-conjugate (Biorad, Hercules, CA, USA) and goat anti-mouse IgG-HRP-conjugate (Biorad, Hercules, CA, USA). Secondary antibody and chemiluminescence procedures were performed according to the instructions of the manufacturer (Perkin Elmer, Wellesly, MA, USA).

Results

The sequences of heavy and light chain gene couples inserted into the pCES-1 vector are shown in Table II. For each DNA segment where codons were missing, we used the germline sequence to fill it up. As a novel and untried method we mixed the primers together in one PCR. After adding the missing codons and the restriction enzyme recognition sites, we cloned the light and heavy chain DNA segments into the pCES-1 vector. We then proceeded to produce fab fragments.

Expression and characterization of recombinant soluble fab fragments with antigen binding sites of B cell receptors from granulomatous lesions

Using *E. coli* TG1 cells, we produced soluble fab fragments from the phage clones that exhibited the specific bind-

(**Table II.** *continued*)

Scu68Vλ

```
                    <---------------------------------------------- FR1 - IMGT
                    1          5          10         15
scu68Vl             cct tct gag ctg act cag gac cct gct ... gtg tct gtg gcc ttg
X56178 IGLV3-19*01  t--- --- ----- --- ----- --- --- --- --- --- ... ---- --- --- ----- ----
                    ----------------------------------------------> _____________
                              20         25         30
scu68Vl             ggg cag aca gtc aca atc aca tgc cag gga gac agc ctc aaa agg
X56178 IGLV3-19*01  ---a ---- --- --- -gg --- --- ----- --a ---- --- ----- --- -g- ---c
                    __ CDR1 - IMGT _____________<------------------------
                              35         40         45
scu68Vl             tat tat ... ... ... ... ... ... gca ggc tgg tat aag cag aag
X56178 IGLV3-19*01  --- --- ... ... ... ... ... ... ---- a--- --- ---c c--- ---- ---
                    FR2 - IMGT ----------------------------> ________ CDR2
                              50         55         60
scu68Vl             cca gga cag gcc cct caa ctt gtc att tat gct aaa aac ... ...
X56178 IGLV3-19*01  --- ---- ---- ---- ---- gt- --- ----- --c --- -g- ---- --- ... ...
                    - IMGT _________ <-----------------------------------------
                              65         70         75
scu68Vl             ... ... ... ... ... agg cgg ccc tca ggg atc cca ... gac cga
X56178 IGLV3-19*01  ... ... ... ... ... -ac ---- ---- ---- ---- --- ---- ... ---- ----
                    ------------------------------------ FR3 - IMGT ---------------
                              80         85         90
scu68Vl             ttc tct ggg tcc aac ... ... tca gga gac aca gcc tcc ttg acc
X56178 IGLV3-19*01  --- --- ---c ---  -g- ... ... --- ----- a--- ----- --t --- --- ---
                    ------------------------------------------------------------> ____
                              95         100        104
scu68Vl             atc act ggg gct cag gcg gaa gat gag gct gaa tat ttc tgt aat
X56178 IGLV3-19*01  --- --- ---- ---- --- ---- ---- --- ---- --- --c -- -a- --- --c
                    _______________  CDR3 - IMGT  _______________
scu68Vl             tcc cgc gac ggc ttt  gaa aat cat cgg gtg ctt ttc ggc gga ggc
X56178 IGLV3-19*01  --- --g ---- a-- ag- -gt --c --- -t

scu68Vl             acc aag ctg acc gtc ctc gag
```

P S E L T Q D P A V S V A L G Q T V T I T C Q G D S L K R Y Y A G W Y K Q K P G Q A P Q L V I Y A K N R R P S G I P D R F S G S N S G D T A S L T I T G A Q A E D E A E Y F C N S R D G F E N H R V L F G G G T K L T V L E

Pr3pos VH

```
                    <---------------------------------------------- FR1 - IMGT
                    1          5          10         5
Pr3posvh            cag gtg cag ctg gtg gag tct ggg gga ... ggc gtg gtc cag cct
M99663 IGHV3-30*03  ---- ---- ---- ---- ---- ---- --- --- ---- ... --- --- --- --- ---
                    ----------------------------------------------> _________________
                              20         25         30
Pr3posvh            ggg agg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc
M99663 IGHV3-30*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    CDR1 - IMGT _____________ <----------------------------
                              35         40         45
Pr3posvh            agt agc tat ggc ... ... ... ... atg cac tgg gtc cgc cag gct
M99663 IGHV3-30*03  --- --- --- ---  ... ... ... ...  --- --- --- --- --- --- --- ---
                    FR2 - IMGT ----------------------------> _________ CDR2
                              50         55         60
Pr3posvh            cca ggc aag ggg ctg gag tgg gtg gca gtt ata tca tat gat gga
M99663 IGHV3-30*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    - IMGT _________ <-----------------------------------------
                              65         70         75
Pr3posvh            agt aat aaa ... ... tac tat gca gac tcc gtg aag ... ggc cga
M99663 IGHV3-30*03  ---- ---- ---- ... ... --- --- --- --- --- --- --- ... --- ---
                    ------------------------------------ FR3 - IMGT ---------------
                              80         85         90
Pr3posvh            ttc acc atc tcc aga gac aat tcc aag aac acg ctg tat ctg caa
M99663 IGHV3-30*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    ------------------------------------------------------------> __
                              95         100        104
Pr3posvh            atg aac agc ctg aga gct gag gac acg gct gtg tat tac tgt gcg
M99663 IGHV3-30*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    _____________________________ CDR3 -  IMGT ______
Pr3posvh            aag agc caa atg tcg tat tac gat ttt tgg agt ggt tat tac cgg
M99663 IGHV3-30*03  -ga ga
                    _____________________________
Pr3posvh            gac cag tac tac ttt gac tac tgg ggc cag gga acc ctg gtc acc
```

Q V Q L V E S G G G V V Q P G R S L R L S C A A S G F T F S S Y G M H W V R Q A P G K G L E W V A V I S Y D G N K Y Y A D S V K G R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K S Q M S Y Y D F W S G Y Y R D Q Y Y F D Y W G Q G T L V T

Table II continues on next page

Table II. *continued*)

Pr3posVλ

```
                    <---------------------------------------------- FR1 - IMGT
                    1              5              10             15
Pr3posvl            cag tct gcc ctg act cag cct gcc tcc ... gtg tct ggg tct cct
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
                    ----------------------------------------->_________________
                                   20             25             30
Pr3posvl            gga cag tcg atc acc atc tcc tgc act gga acc agc agt gac gtt
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    CDR1 - IMGT _____________ <--------------------------
                                   35             40             45
Pr3posvl            ggt ggt tat aac tat ... ... ... gtc tcc tgg tac caa caa cac
Z73664 IGLV2-14*01  ---- --- --- ---- --- ... ... ... --- ---- ---- --- ---- --g ---
                    FR2 - IMGT ----------------------------> _______ CDR2
                                   50             55             60
Pr3posvl            cca ggc aaa gcc ccc aaa ctc atg att tat gat gtc agt ... ...
Z73664 IGLV2-14*01  ---- --- ---- --- ---- --- --- --- --- --- --g --- --- ... ...
                    - IMGT ________ <-----------------------------------------
                                   65             70             75
Pr3posvl            ... ... ... ... ... aat cgg ccc tca ggg gtt tct ... aat cgc
Z73664 IGLV2-14*01  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
                    ---------------------------------- FR3 - IMGT ---------------
                                   80             85             90
Pr3posvl            ttc tct ggc tcc aag ... ... tct ggc aac acg gcc tcc ctg acc
Z73664 IGLV2-14*01  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
                    ------------------------------------------------------------> ___
                                   95             100            104
Pr3posvl            atc tct ggg ctc cag gct gag gac gag gct gat tat tac tgc agc
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    _______ CDR3 - IMGT ______
Pr3posvl            tca tat aca agc agc agc act gaa gtg ttc ggc gga ggg acc aag
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- ctc

Pr3posvl            ctg acc gtc ctc gag
```

Q S A L T Q P A S V S G S P G Q S I T I S C T G T S S D V G G Y N Y V S W Y Q Q H P G K A P K L M I Y D V S N R P S G V S N R F S G S K S G N T A S L T I S G L Q A E D E A D Y Y C S S Y T S S S T E V F G G G T K L T V L E

The nucleotide sequences of two VH-Vλ couples used for Fab-generation. "PR3pos" is a fab based on the WGH1-sequence (22) which encodes for an antiPR3-IgM-λantibody (kindly provided by Dr. Csernok, Bad Bramstedt, Germany). The IMGT database was used for sequence-comparison (http://imgt.cines.fr). The single cell sequences each are aligned to their respective germline gene. Homolgue nucleotides are symbolized by "-". FR: framework regions and CDR: Complementarity determining regions are indicated by bars. Nucleotide positions are provided. The resulting amino acid sequences of the fabs are shown below the nucleotide sequence alignment.

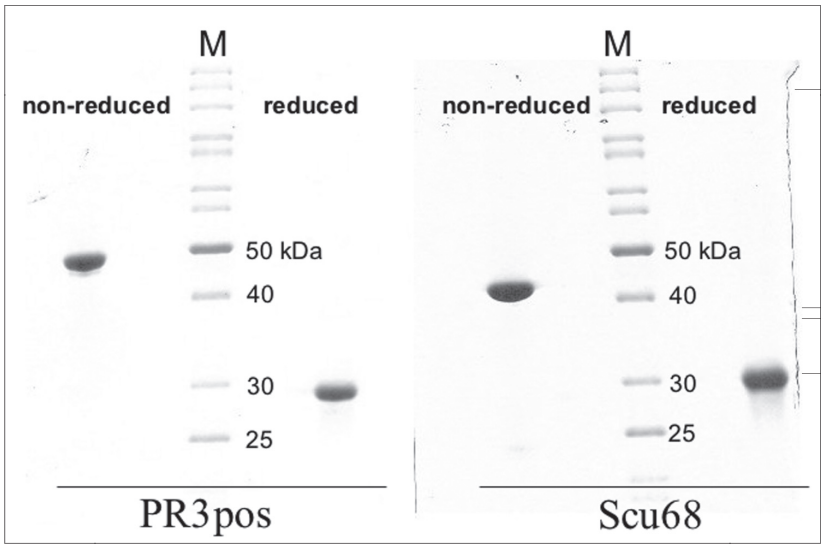


Fig. 2. Expression and purification of fab fragments with antigen binding sites of B cell receptors from granulomatous lesions. SDS-PAGE analysis of reduced- and nonreduced-purified Fab protein after metal affinity chromatography. Two represesentative clones are shown, PR3pos and Scu68 respectively. (M) indicates the marker, the molecular weight of bands is depicted.

ing pattern of antibodies encoded by B cells from WG granulomatous lesions. These Fab fragments were purified by metal affinity chromatography from the periplasm using the hexahistidine tag fused to the CH1 domain of the fab fragments. SDS-PAGE analysis of the affinity-purified material revealed homogenous, pure fab antibodies with the expected molecular weight (Fig. 2). The non-reduced fab protein migrates at approximately 50 kDa. Under reducing conditions the disulfide bond is dissolved, which connects light and heavy chain to a heterodimeric protein. Under these conditions a band at approximately 30 kDa becomes visible, when the gel is stained with coomassie. Two representative clones are shown, PR3pos and Scu68, respectively. Approximately 0.2-1 mg of pure material could be obtained from 1 liter of bacterial culture. Next we confirmed the heterodimeric nature of the fab fragments. By western blot the light chain can be visualized with a specific antibody. The fragment of the heavy chain was visualized through detection of the hexahistidine tag (Fig. 3).

Discussion

The analysis of immunoglobulin genes from B cell-rich lymphatic structures in WG granulomata revealed clonal expansion. This is indicative of a germinal centre-like reaction, where antigen-driven affinity maturation takes place. Our hypothesis is that ANCA-producing B cells are formed in the granulomata by an antigen-driven process. A candidate antigen is PR3 which is highly expressed within these germinal centre-like structures (12).

The WG fabs were derived from characteristically mutated immunoglobulin genes in order to draw nearer to the structure function relationship that is driving the affinity for a yet unknown target antigen. For positive control we created a fab based on the sequence of WGH1, an established antiPR3 antibody (22).

Most of our WG single B cell DNA segments coding for the heavy chains were incomplete. We fixed this problem by supplementing these DNA segments with germline DNA and added the restriction enzyme recognition sites for

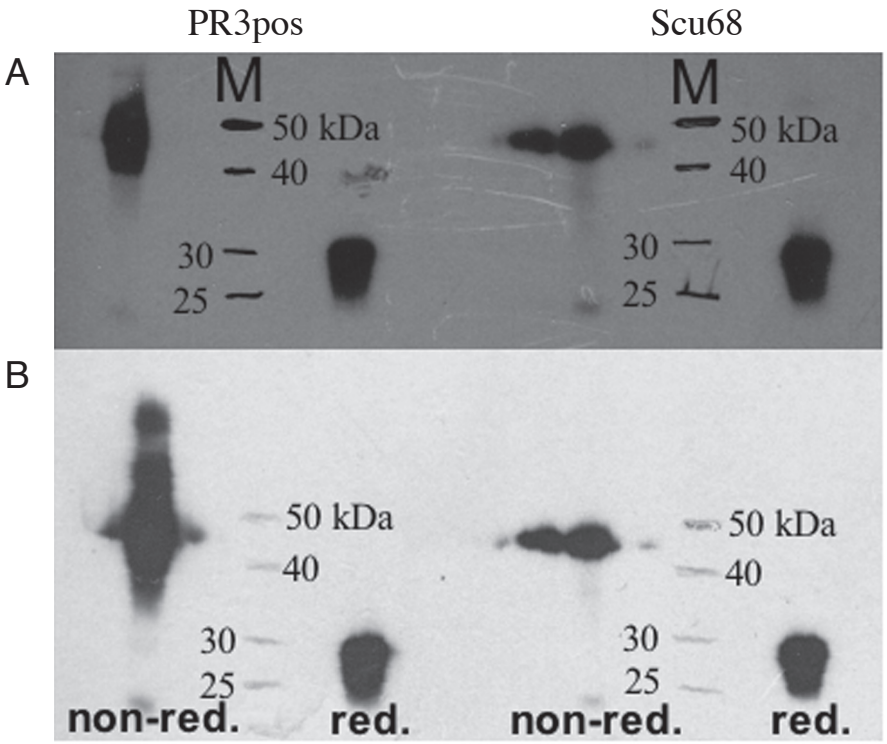


Fig. 3. Fab fragments are heterodimeric proteins. Reduced and non-reduced soluble, recombinant fab fragments of the indicated clones were seperated by SDS-Page and stained by western blot. The identical membrane was first stained with an anti-human IgG (**A**), stripped and thereafter stained with an anti-Penta-His IgG (**B**). (M) indicates the marker, the molecular weight of bands is depicted.

cloning into the pCES-1 vector. This meant adding more nucleotides then is possible in one PCR reaction. As an experiment we mixed the different primers, which were necessary in each reaction, together and performed the PCR reaction in one step. This technique of mixing primers resulted in a slight reduction of time and labour. We succeeded in cloning all of the DNA coding for the variable domain of the heavy and light chains into the pCES-1 vector. This allowed us to produce fab fragments representing an antibody, which is encoded by B cells from granulomatous lesions of WG. Characterization of their target will help in elucidating the selection and maturation process of these B lymphocytes. The discovery of potential antigens allows understanding of the initial pathogenesis of this disease and might help to determine the origin of ANCA as well as the mechanisms driving the expansion of selected B cells. Furthermore, knowledge of the antigen opens a new category of therapy, since abrogation of the antigen or blocking its interaction with the B cell receptors might interrupt the deleterious inflammatory process.

In conclusion this is the first study, which describes a methodology for the generation of antibody-like structures, the fab fragments, derived from single B cells of WG lesions. Further work is currently being undertaken to characterize the target antigen and binding properties of these fab fragments.

Acknowledgements

We thank Prof. Wolfgang L. Gross, University of Luebeck and Rheumaklinik Bad Bramstedt, Germany, for support and critical discussion of the matter. We thank Dr. Elena Csernok for kindly providing a WGH1 hybridoma. We thank Gabriele Carbon and Birgit Bette from the José Carreras Center for Immunotherapy, Homburg, Germany, for technical assistance and Dr. Dieter Preuss for helpful discussion of fab generation and testing.

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