Development of a System to Test for Specificity of B-cell Receptors Found in Granulomatous Lesions of Wegeners Granulomatosis patients

A Proposal for an M.D Study by A.J. Kerkdijk

Submitted to the

University of the Saarland, Germany, for the degree of Doctor of Medicine

Supervisor: Prof. Dr. M. Pfreundschuh

1. Summary

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 identified 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.

The results of this study have been presented and published in:

1) J. Voswinkel, A.J. Kerkdijk, A. Mueller, G. Assmann, M. Pfreundschuh, G. Held. A novel system to test for specificity of B-cell receptors from tissue of Wegener's granulomatosis patients. Clin Exp Rheumatol 2008; 26 (Suppl. 49): S90-S96. [32]

2) Kerkdijk AJ, Held G, Mueller A, Gross WL, Pfreundschuh M, Voswinkel J: Development of a System to Test for Specificity of B-cell Receptors found in Granulomatous Lesions of Wegeners Granulomatosis patients. 37th annual meeting of the German Society for Immunology, Heidelberg.

2. Introduction

2.1 Immunological background information

Secondary lymphoid structures include Peyer's patches and the mucosa-associated lymphoid tissues. Environmental stimuli can promote the development of these secondary lymphoid structures from clusters of lymphoid tissue-inducer cells and DCs in the lamina propria, the so-called "cryptopatches"[5]. Tertiary lymphoid structures are ectopic accumulations of lymphoid cells found in locations which are non-lymphoid and are very similar to secondary lymphoid organs. For instance, specialized high-endothelial venules have been detected[5]. How tertiary lymphoid structures arise is unknown, but it is probable that chronic cytokine expression plays a role as tertiary lymphoid structures have been found in diseases like Hashimoto's thyroiditis, rheumatoid arthritis, ulcerative colitis and Sjögren's syndrome[5]. In these tertiary lymphoid structures immunohistochemical analysis showed the presence of germinal centers[5]. The lymphoid structures which are found in the granuloma-like formations of Wegener's granulomatosis also show a histological pattern consistent with a germinal center and can therefore also be considered lymphoid like structures[20,33].

2.2 Characteristics of Wegener's granulomatosis

Wegeners granulomatosis is a disease entity with three important clinical characteristics[27]:

1. Necrotising granulomatous inflammation of the respiratory tract
2. Pauci-immune Glomerulonephritis
3. Systemic small-vessel vasculitis

At the Chapel Hill International Consensus Conference in 1992, Wegener's granulomatosis was defined as:

Granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium sized vessels (e.g., capillaries, venules, arterioles, and arteries)[12]

"Classic" Wegener's granulomatosis takes a biphasic course[16]. The initial phase is mainly characterized by granulomatous inflammation of the upper and lower respiratory tract, but

granulomatous manifestations of disease are possible in virtually every organ. After a variable amount of time generalization takes place and problems associated with glomerulonephritis and systemic vasculitis evolve. Once the disease has generalized, untreated patients show a rapidly progressive fatal disease course[10], which necessitates immunosuppressive treatment.

As a result of the changing nature of the disease over time, the European Vasculitis study Group (EUVAS) has defined the following disease stages, based upon clinicopathological considerations.

Table 1[1]: Definition of disease stages by the European Vasculitis Study group (EUVAS) by clinico-pathologic criterias. It was agreed that the level of immunosuppression should reflect the severity of vasculitis.

Clinical subgroup	Constitutional symptoms	ANCA status	Threatened vital organ functions
Localized	No	Negative/positive	No
Early systemic	Yes	Positive	No
Generalized	Yes	Positive	Yes
Severe renal	Yes	Positive	Yes
Refractory	Yes	Positive/negative	Yes

Localized disease refers to the granulomatous manifestations of the disease in various organ systems, of which the respiratory tract is most often affected without signs of vasculitis.
Early systemic WG refers to the disease stage which is characterized by signs of vasculitis without renal involvement or vital organ failure. Generalized WG refers to the systemic disease with renal involvement.

2.2.1 Clinical characteristics

As mentioned above, WG can be divided into a localized and generalized, systemic form.
Approx. 42% of patients are diagnosed within 3 months of symptom onset, but a correct diagnosis might take years to be established[10]. Ninety percent of patients first seek medical care because of upper or lower airway symptoms[10].

Below, we will discuss the disease manifestations which can occur in various organ systems based upon a number of review articles and large long-term studies of WG patient cohorts [10,16,25].

Localized disease

Upper airway manifestations based upon granulomatous inflammation include nasal obstruction caused by mucosal swelling, crusting and bloody nasal discharge or epistaxis and in advanced disease stages necrosis of the nasal cartilage with subsequent perforation of the nasal septum[15], a complication which might not become clear initially because of the mucosal swelling [1]. Another consequence of this necrosis is the saddle-nose deformity, see the figure below. Further, paranasal sinus involvement is possible with chronic sinusitis and consequent headache, serosanguineous discharge and chronic cough and nasal congestion[1]. Otitis media, mastoiditis and hearing loss might also occur[15]. At diagnosis, 93% of patients have upper respiratory tract symptoms[25].
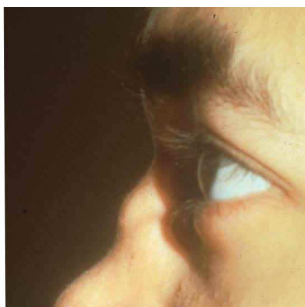
Figure 1:
Saddle nose deformity,
permission for reproduction kindly
granted by Dr. Stephen Foster

Lower airway manifestations of WG include subglottic stenosis, which occurs in 10-20% of WG patients and occurs in 8% of patients at presentation[25]. Eighty-five percent of WG patients eventually develop lung disease throughout the course of their disease[10]. According to one study roughly 45% of patients present with pulmonary infiltrates, nodules or both at initial presentation. Using CT-scans these percentages are even higher, 50-70% of patients

have abnormal chest CT-scans[1]. Bronchial ulcers as a result of granulomatous inflammation might also occur. Symptoms which might arise are cough, hemoptysis and pleuritis[10].

Generalized disease

Generalization is the occurrence of systemic vasculitis. Upon generalization constitutional and rheumatic complaints occur: malaise, fever, night sweats, weight loss and polymyalgia, arthralgia or arthritis[16], these symptoms announce generalization or relapse of disease.

Renal involvement ranges from asymptomatic abnormalities in the urine sediment to the pulmonary-renal syndrome.

The **pulmonary-renal syndrome** is the most serious consequence of the generalized vasculitis with rapidly progressive necrotizing glomerulonephritis resulting in renal failure and alveolar hemorrhage with very high mortality if untreated[16].

2.2.2 Epidemiology of Wegener's granulomatosis

The prevalence of WG is an estimated 23.7 – 156.5 per million[18], the combined annual incidence of the ANCA-associated, predominantly small-vessel vascultides Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome is 10-20 per million[34]. There seems to be a north-south gradient for the incidence of WG with the highest incidence in the Scandinavian countries[34], suggesting an environmental factor involved in the pathophysiology of WG, see table 2.

Table 2. Incidence of WG in Europe (per million population)

Norway	10.5
UK	10.2
Germany	8.0
Spain	4.9

Population based surveys indicate a mean age of onset which lies between 50 to 66 years[18]. There is some evidence for a slight male predominance in WG, but the evidence is inconclusive[18].

WG seems to be less prevalent in non-whites; a study from France found a prevalence for WG which was half as high in non-Europeans from Africa, Asia and the Caribbean as compared to whites[18,34]. There have been a number of case-control studies which link silica-exposure to risk of WG with an odd-ratio of 2.5-5[18]. Another important environmental factor is chronic nasal carriage of Staph. aureus, which will be discussed in more detail below.

2.2.3 Anti-neutrophil cytoplasmic antibodies (ANCAs)

There is a subgroup of small-vessel vasculitides which are ANCA-associated:

1. Wegener's granulomatosis
2. Microscopic polyangiitis
3. Churg-Strauss syndrome

Anti-neutrophil cytoplasmic antibodies (ANCAs) probably play a very important role in the pathogenesis of Wegener's granulomatosis. They are IgG antibodies and there are two ways to detect the presence of these antibodies. The first way is through indirect immuno-fluorescence (IIF). Ethanol-fixed neutrophils from healthy donors are incubated with patient serum and two distinct staining patterns can be recognized. There is the cytoplasmic staining pattern where most of the IgG which is not washed away in the test stains cytoplasmic components of the neutrophils. This staining pattern is diagnostic for the presence of cANCAs (ANCAs which mainly stain the cytoplasm). Another staining pattern which can be recognized is the perinuclear pattern, where staining mainly occurs around the nucleus, proving the presence of pANCAs (ANCAs which mainly stain the perinuclear portion of the cytoplasm). See figures 2 and 3 for examples.
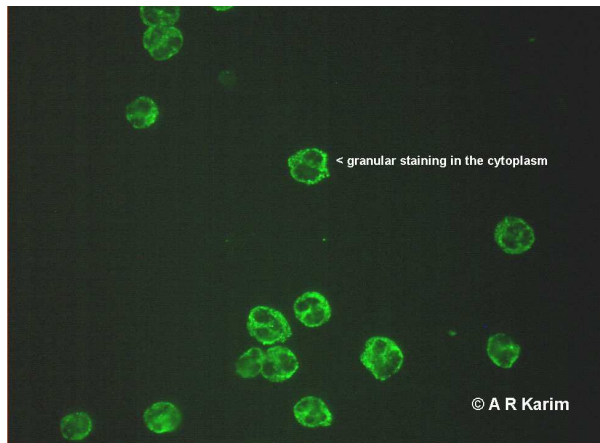
Figure 2:

cANCA staining pattern by IIF,
permission for reproduction kindly
granted by Dr. A.R. Karim
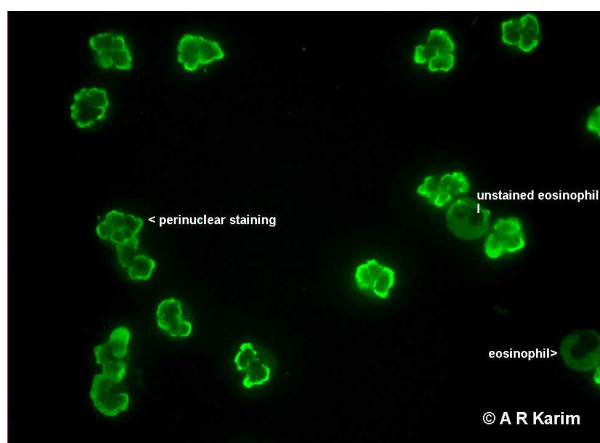
Figure 3:

pANCA staining pattern by IIF,
permission for reproduction kindly
granted by Dr. A.R. Karim

Most of the cANCAs and most of the pANCAs are directed against proteinase-3 (PR-3) and myeloperoxidase (MPO) respectively[2,17]. This is the basis for the second diagnostic test used in the ANCA-associated small vessel vasculitides: ELISA to check for the presence of anti-PR3 and anti-MPO antibodies in the serum.

In the following table the sensitivity of indirect immunofluorescence and ELISAs for the three ANCA-associated vasculitides is presented:

Table 3: Sensitivity of ANCAs for ANCA-associated vasculitides[9]

	cANCA IIF	pANCA IIF	cANCA or pANCA IIF	Anti-PR3 ELISA	Anti-MPO ELISA
Wegener's	64%	21%	85%	66%	24%
Churg-Strauss-syndrome	33%	33%	66%	17-33%	50%
Microscopic polyangiitis	23%	58%	81%	27%	58%

As can be recognized from this table, WG is mainly associated with cANCAs and MPA is mainly associated with pANCAs, although there is some overlap.

The specificity for the ANCAs in disease and healthy controls is shown in the following table. Disease controls include patients with other forms of vasculitis, other glomerulonephritis patients, patients with connective tissue diseases and other granulomatous diseases.

Table 4: Specificity of ANCAs determined using IIF and ELISA in patients and healthy controls[9]

	cANCA IIF	pANCA IIF	cANCA or pANCA IIF	Anti-PR3 ELISA	Anti-MPO ELISA
Disease controls	95%	81%	76%	87%	91%
Healthy controls	98%	96%	94%	98%	96%

From these data it is clear that neither test gives a satisfying sensitivity or specifity, especially the specificity in the group of disease control patients is suboptimal. This problem can be solved by combining the IIF cANCA test and the anti-PR3 ELISA. This combination yields a 56% sensitivity for WG, which is roughly 10% lower than the sensitivities for each separate test, but with an almost perfect specificity of 99% in the group of disease-control patients and a 100% specificity for the healthy controls[9]. The sensitivities for the other two diseases in the

group of the ANCA-associated vasculitides are much lower and the disease control group does not include these other two diseases. Therefore, to delineate WG from these other two diseases, clinical and histological data has to be combined. Still, these tests are powerful tools in the hands of the clinician, especially to exclude disease.

2.2.4 Proteinase-3 (PR3)

Proteinase-3 is a serine proteinase found in azurophilic granules of neutrophilic granulocytes. It has a size of about 29 kDa and preferentially attacks peptide bonds having small aliphatic amino acids (alanine, valine, leucine and serine)[8,24]. The antimicrobial activity is retained following denaturation and even peptide fragments still possess antibiotic activities. The mechanism of antimicrobial action therefore is not fully understood[8]. In healthy conditions the protein is inhibited by alfa-1-antitrypsin. This inhibition is inhibited by the binding of cANCA to PR3 which results in uncontrolled protease activity and might explain part of the pathophysiology in the ANCA-associated vasculitides[2,8]. The cANCAs bind near the active site of PR-3 which inhibits proteolytic activity. The sum of these effects is an increase in proteolytic activity[2].

PR3 remains intracellularly under normal conditions, and is only expressed on the cell-surface of neutrophils and endothelial cells in an inflammatory environment.

PR-3 degrades a variety of matrix proteins including fibronectin, laminin, vitronectin and collagen type IV[24].

Crystal structure analysis has shown that PR-3 forms tetramers[8].

Binding of ANCAs to PR3

ANCA bind to PR3 on neutrophils with their F(ab')2 portion and to neutrophil Fc-gamma receptors IIa and IIIb via the Fc portion[2,23]. ANCA-induced neutrophil activation requires both these receptors to be engaged[2,27].

2.3 Pathogenesis

2.3.1 Fienberg's hypothesis

The Fienberg hypothesis states the biphasic nature of the clinical course of WG on a pathophysiological background: WG starts as a granulomatous disease out of which the systemic vasculitis develops[1]. The earliest lesions in the respiratory tract are foci of swollen collagen fibers, representing a small nidus of necrosis, out of which the granulomatous lesions arise. The strongest argument for this theory is the chronological course of the events in WG: first there is the granulomatous lesion, and the systemic vasculitis usually follows after a variable span of time. Oppononents of this theory might claim that these early foci of fibrinoid necrosis were a consequence of necrotizing capillaritis[1]. This basically is the argument that the vasculitis is the origin out of which the granuloma arises.

Some very important observations have substantiated Fienberg's theory and an extension of this theory forms the basis of this doctoral thesis. There are indications that the granulomatous lesions represent ectopic lymphoid tissue which serves as a breeding-ground where the B-cells which produce PR3-ANCAs arise. The evidence for this hypothesis comes from the following observations[33]:

1. B cell clusters can be found within granulomatous formations from endonasal biopsy specimens of WG patients
2. Analysis of the B cell receptor coding regions showed evidence of a high mutational frequency and clonal expansion, which is characteristic of a germinal center, in which antigen-selection takes place
3. Plasma cells, PR3 and neutrophils expressing PR3 were found in the vicinity of the B-cell clusters

These observations give rise to the possibility that the granulomatous lesion is a lymphoid tissue where affinity-maturation takes place on an unknown antigen resulting in B cells with PR3 affinity and could present the "missing-link" between localized, granulomatous disease and systemic, ANCA-associated disease. In some patients of this study[33] there was a skewing of mutations towards negatively charged amino-acids, consistent with a maturation process on the positively charged PR3-protein.

2.3.2 Evidence for a pathogenic role of ANCAs in WG

A number of studies have appeared during the last 15 years providing evidence for a pathogenic role of ANCAs in the pathogenesis of WG. Since the glomerulonephritis which causes morbidity in WG is a pauci-immune glomerulonephritis, the damage to the glomerulus cannot be explained by deposition of immune complexes. A very important clue to what the pathogenesis of WG might be came from a study by Ronald J. Falk[6] in 1990. He incubated normal human neutrophils with ANCA-positive sera. He made the following discoveries:

1. The release of reactive oxygen species (ROS) from neutrophils was increased after incubation with ANCA-sera and with purified ANCA-IgG as compared to normal sera.
2. Superoxide release was noted only when neutrophils were primed with TNF before incubation.
3. Primary granule degranulation was noted after incubation with ANCA IgG, but only after priming with TNF.
4. When neutrophils were primed with TNF flowcytometry analysis showed that MPO was expressed at the cell surface.

The latter finding could explain the fact that superoxide release and primary granule degranulation only take place after priming with TNF. Priming obviously makes the antigen for the ANCAs available at the cell-surface so neutrophils can be activated, whereas in normal neutrophils the antigen is not available because it is stored in secretory granules within the cell.

This very important study gave a first idea about the pathogenesis of pauci-immune vasculitis, in the absence of immunecomplexes in the glomerulus. In this model neutrophil priming takes place through an unknown factor, for instance an infection, after which the neutrophils degranulate in the presence ANCAs and set free reactive oxygen species which cause vasculitis.

In vivo evidence for a role of ANCAs in the pathogenesis of WG was provided by a pivotal study by Xiao et al[35].

In the Xiao study MPO-knockout mice were immunized with either MPO or BSA and anti-MPO antibody (MPO-ANCA) titers developed in MPO-immunized mice. Splenocytes from both groups of mice were injected into RAG2-/- mice. These mice lack functioning T- and B-cells. All mice receiving splenocytes developed urinary abnormalities, but only mice who received anti-MPO splenocytes developed severe necrotizing and crescentic glomerulonephritis as well as segmental or global glomerulal necrosis. None of the mice who received anti-BSA or control splenocytes developed glomerular crescents. Furthermore, of the 16 mice who received the anti-MPO splenocytes one developed necrotizing arteritis in spleen and lymph nodes, one necrotizing arteritis in the lungs and five developed hemorrhagic pulmonary capillaritis. To ascertain that these effects were really due to the antibodies and not because of T-cells which are present in the splenocytes five RAG2-/- and six wild-type mice were injected with purified anti-MPO IgG or anti-BSA IgG. All five rag2-/- mice had focal necrotizing glomerulonephritis and crescents, none of the three mice which received anti-BSA IgG had histologic lesions. The same was true for wild-type mice. Focal pulmonary alveolitis was seen in two of the six wildtype mice that received anti-MPO IgG. The glomerulonephritis which was induced by the anti-MPO splenocytes and anti-MPO IgG was similar to the pauci-immune glomerulonephritis seen in humans with small-vessel vasculitis.

The Xiao study was the first in vivo indication that MPO-ANCA might have a causative role in the development of the pauci-immune glomerulonephritis seen with the ANCA-associated vasculitides.

The role of ANCAs in the pathogenesis of WG can be summarized in the following picture which shows neutrophil priming, possibly by an infection leading to release of proinflammatory cytokines, upregulation of adhesion molecules on both neutrophils and endothelial cells and ANCAs binding to neutrophils with both their Fc-portions and their antigen-binding site leading to degranulation which sets free reactive oxygen species which causes endothelial damage, which translates to vasculitis.
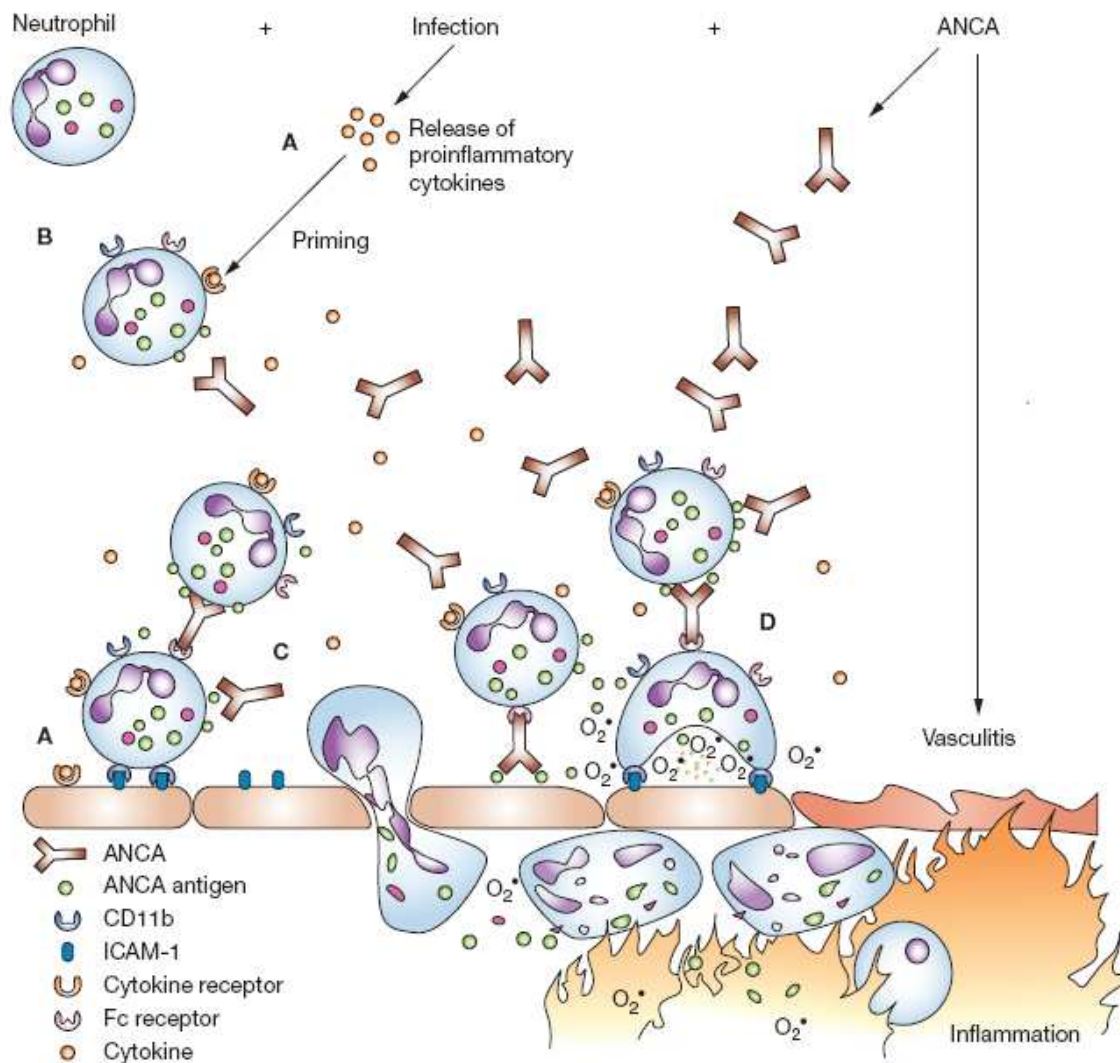
Figure 4:

Role of ANCAs in pathogenesis of vasculitis in WG[13]

Association with Staphylococcus Aureus

There is an association between chronic nasal carriage of Staph. aureus and relapse rates in WG. In one study[30] 22 of 33 patients with chronic nasal carriage had a relapse as compared to only 1 of 21 persistently negative patients, the relative risk was 7.16 with a confidence interval of 1.63 to 31. Treatment with co-trimoxazole leads to a significant reduction in respiratory tract and other infections and leads to a significant reduction in the incidence of relapse in WG patients who are in remission[29]. Furthermore, chronic nasal carriage of Staph. aureus is three times higher in WG patients as compared to healthy people[22]. Staph. aureus is known for its potent immunostimulatory effect[22]. This effect mainly comes from its exotoxins, which act as superantigens. These superantigens are able to stimulate T cells

16

unspecifically, that is they can bind to MHC class II molecules outside the peptide-binding groove and still activate the T-cell[22]. Staphylococcus Aureus might play an important role in the initiation of inflammation in the respiratory tract, after which a granulomatous lesion is formed. Also, it might act as an antigen with similarity to PR3 leading to crossreactivity and formation of the PR3-ANCA. Interestingly, there are B cell superantigens like SPA and SED, present in staph. aureus bacteria, that activate B cells via conserved structures of IgG without T-cell help, which could, within the granuloma-like structures of Wegener's granulomatosis, lead to B-cell expansion. Of note, the B-cells which can be stimulated by these superantigens are of the VH3-23 immunoglobulin family and this family was also predominantly found in our analysis of B-cells derived from granuloma-like formations of Wegener's patients.

2.4 Hypotheses and goals of this study

Our hypothesis is that formation of the cANCA producing B-cells takes places within the granulomatous lesions seen in Wegener's granulomatosis and that PR3 is the antigen on which this affinity maturation takes place. Other possible candidates for the antigen on which the apparent affinity maturation takes place are epitopes of Staph. aureus. Staph. aureus colonisation of the upper respiratory tract is associated with higher relapse-rates in Wegener's granulomatosis and therefore seems to have a role in pathogenesis. To prove our hypothesis we aimed to define the specificity of the B-cell receptors from B-lymphocyte-rich clusters within granulomatous lesions. The goal of this study was to devise a system which enables us to investigate the specificity of the B-cell receptors found in the aforementioned granulomatous lesions, possibly giving new clues to the pathogenesis of Wegener's granulomatosis. To achieve this goal we isolated single B-cells from the aforementioned granulomatous lesions and using the DNA isolated from these B-cells set out to produce Fab fragments. A Fab fragment is an antibody lacking the Fc-fragment, while preserving the highly variable antigen-binding part of the antibody. We modified the Pces1 vector in such a way that the separate cloning of DNA coding for variable heavy and light-chain becomes possible. Intact Fab fragments based on the isolated B-cells were produced, which can then be used as an antibody analogue, for specificity testing.

2.5 Preliminary work

The material which was the basis for the present study was the result of preliminary work performed in the laboratory of Rheumaklinik Bad Bramstedt, Lübeck. In summary, nasal biopsy specimens of two WG patients were obtained. If the tissues showed the typical granulomatous formations of WG they were immunohistochemically treated with anti-CD20 to identify B-cells. Individual B-cells from these granulomatous formation which were located in the aforementioned germinal-center like structures were isolated using a laser technique as described by Fink et al.[7] and Küppers et al.[14]. This is shown in the following pictures.
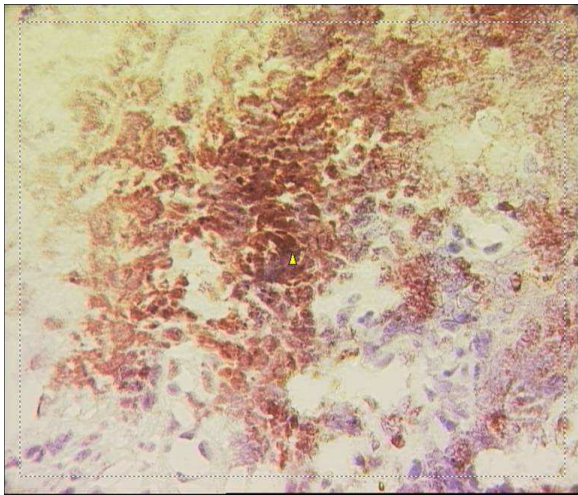
Figure 5:

Laser-assisted capture of a single

B-cell from a granulomatous lesion
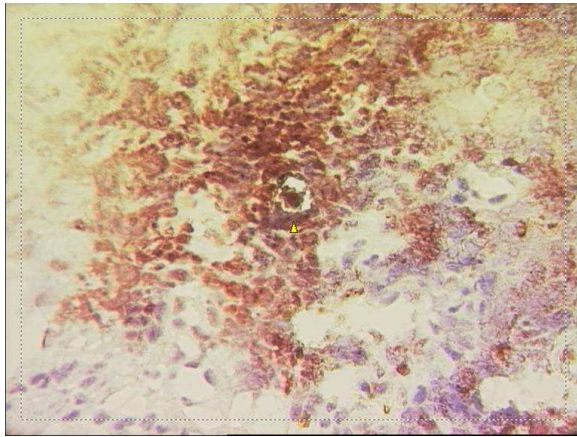
Figure 6:

Laser-assisted capture of a single
B-cell from a granulomatous lesion
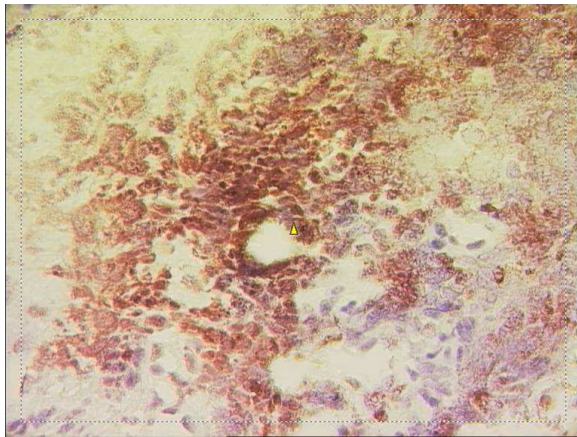
Figure 7:

Laser-assisted capture of a single B-cell from a granulomatous lesion

The result of this laser technique was the isolation of individual B-cells. These individual B-cells each contain only one copy of the entire genome, including the rearranged VDJ segment which codes for the B-cell receptor. Thus, this is a very small quantity of DNA. We characterized the VDJ segment of the variable domain of both the light and heavy chain of the B-cell receptor, in order to construct Fab-fragments. It is possible to amplify these DNA segments by using PCR techniques, as described below. To perform PCR, primers need to be constructed which are specific for the different types of V and J segments. However, beforehand it is unknown which V and J family is present in the excised B-cell, as the V and J segments are available in multiple copies in the genome. Consecutively two rounds of PCR were performed where in the first round a mixture of all primers specific for the different "families" of V-segments was used combined with a mixture of primer specific for the

different J-segments which are available for the variable domain of the heavy chain and both λ- and κ- variety of the light chain. Whatever combination of V(D)J segment exists in the B-cell, it is amplified by this PCR because all possible VDJ-segment combinations are represented by the primers we employed. After this first round of PCR the VDJ segment has been amplified but it is still unknown to which "family" the V segment belongs. Therefore, a second round of PCR was performed where the DNA resulting from the first round of PCR was used to perform a series of PCRs where in each individual PCR only one primer, specific for only one family was used. The product of each PCR was run through a gel using electrophoresis. If a DNA-product was identified, the specific "clan" to which the V segment used in that B-cell belongs, has been determined, as it is equal to the clan for which that primer is specific. For instance, there are about 40 different copies of the V segment of the heavy chain, which can be grouped, on the basis of sequence-homology into about six "families". Six different primers specific for each family can be used to make six different PCRs on DNA of a single B-cell. The differences between the primers are large enough so only one primer will bind to the DNA and yield a DNA product on gelelectrophoresesis, which is shown in the following picture where six different PCR products resulting from PCRs performed with the six different primers are displayed. In this case the variable segment of the heavy chain belongs to the VH3-family.
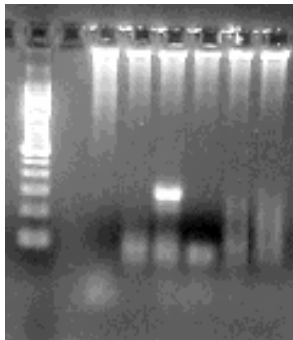
Figure 8:

Second round PCR (see text for explanation of first and second round PCR)

The product in the third lane, shown in the picture above, was excised from the gel and this was also done for both the heavy and light chains, yielding two PCR products for each single B-cell which was isolated from the granulomatous formations of WG patients.

This procedure was done for two B-cells from two different patients. For each cell a light- and heavy chain PCR product was isolated from the gel, as described above. The following table shows the designations which were given to the B-cells isolated from the two patients (St, and Scu), along with the light chain.

Table 5: Names of two B-cells derived from two WG patients and their light-chain subclass

B-cell	Light chain
St140	κ
Scu68	λ

2.6 Phage display technology

As described in "hypotheses" we wanted to produce a Fab fragment. To achieve this goal we used phage display technology.

Introduction

Phage display is a technique to investigate protein-protein interactions. Recombinant proteins are displayed on the surface of phage particles and these phage particles can be used to test binding of these recombinant proteins to other proteins. This can be done through "panning experiments" where many different phage particles with many different recombinant proteins are brought together with a protein which is bound on an ELISA plate so that phage with recombinant protein and the target-protein on the plate can engage in interaction and bind to eachother. Non-bound phages are washed off, and this selection process, which is called "panning", can be repeated to enrich the percentage of target protein binding phages after each round (see figure 9 for the principle). The advantage of phage display is that the phage particles display the protein on their surface and within the phage is the DNA which codes for this protein. It is thus very easy to find out which recombinant protein bound to the target-protein by DNA-analysis. A second advantage is that phage can be used to display millions of different proteins on their surface in a relatively easy way, thereby allowing the screening of millions of different proteins for binding-capability to the target-protein.
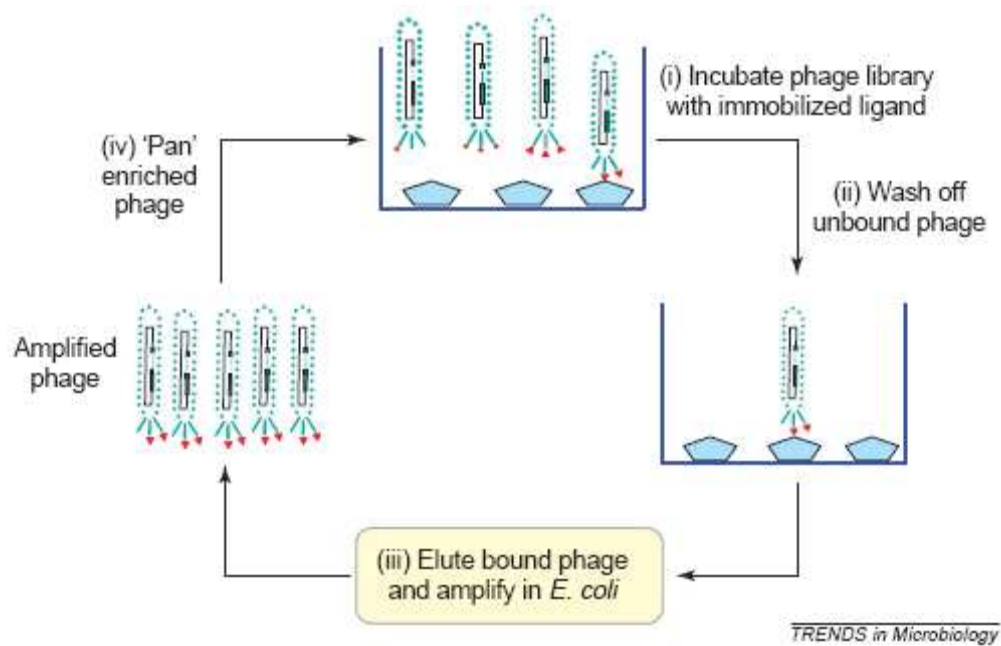
Figure 9:

The principle of panning using recombinant proteins displayed on phage[19]

This technique has been used to investigate host-pathogen interactions, receptor-ligand interactions, vaccine development and, very importantly, antibody-antigen interactions.

Various phage can be used in phage display. The fd filamentous phage is used frequently in phage display and is shown in figure 10. Basically, an fd phage is composed of a circular single-stranded piece of DNA surrounded by a coat protein and four additional types of proteins at both ends of the phage. The DNA codes for these proteins.
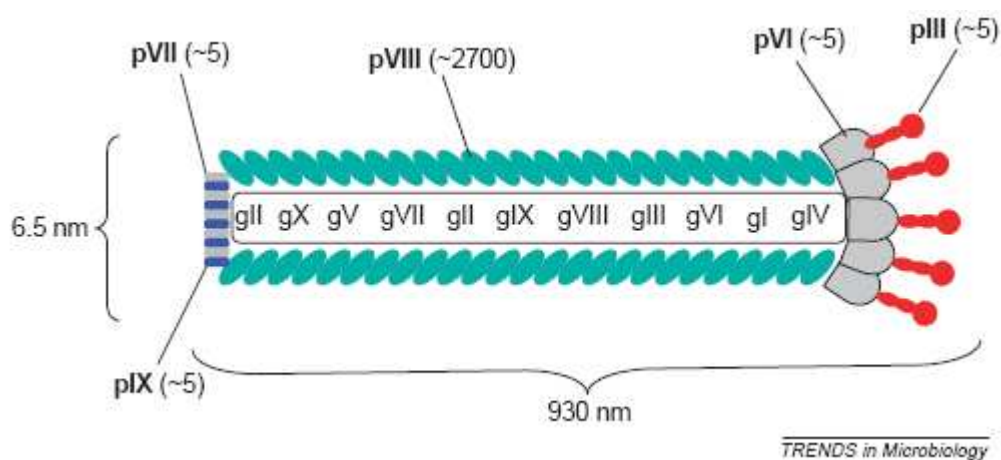
Figure 10:

Filamentous phage fd[19]

Fd phage infect E. coli through protein III (pIII) which adheres to the F pilus of F+ strains of E. coli. This causes loss of the F-pilus which prevents superinfection. Recombinant protein can be expressed as a fusion protein with pIII through combination of the phage DNA with DNA coding for the recombinant protein. The pIII consists of an N-terminal domain, binding to the F-pilus and a C-terminal domain which is buried in the particle. Both domains are about 200 amino acids long. A foreign (recombinant) protein can be fused to the pIII at the N-terminal end which causes the recombinant protein to be expressed at the tip of the pIII, which is necessary of course to engage in a possible interaction with the target protein.

The life cycle of the fd phage is shown in figure 11. Shortly: the fd phage binds to the F-pilus and injects its single-stranded DNA into the E.Coli. Double-stranded DNA is synthesized from this single-strand and gene transcription and protein synthesis start from this DNA. Eventually new virions are created as the DNA is extruded across the cell membrane where it combines with the hull proteins which have accumulated at the cell membrane to form new phage which can then re-infect other cells. Note that fd phage is a non-lytic phage, that means it does not kill the bacteria it infects which makes it a little bit more difficult to "harvest" protein which have been synthesized after infection. These proteins have to be harvested from the periplasm where these proteins accumulate, see below.
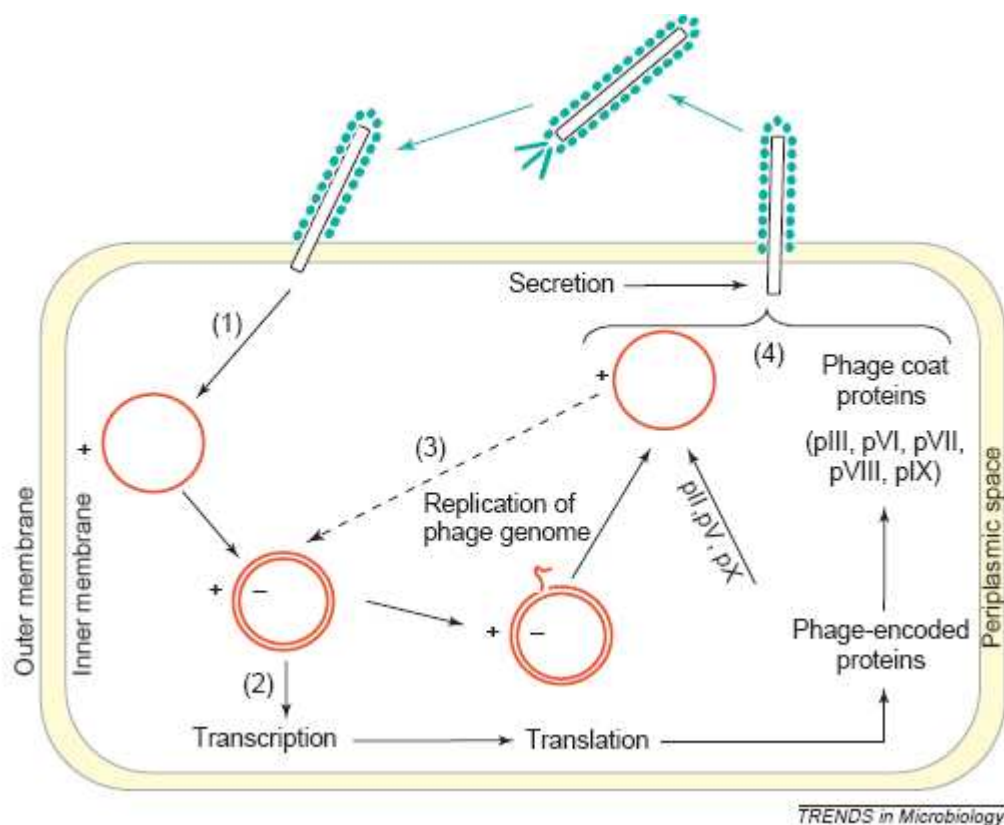
Figure 11:
Life cycle of filamentous phage[19]

Methods have been devised which allow the cloning of millions of different recombinant proteins into phage DNA. To facilitate this, a so-called phagemid has been constructed. A phagemid is a hybrid of phage DNA and a normal plasmid vector. Because the phagemid can be handled as a normal plasmid, the standard parallel transformation (see "transformation") of millions of different DNA-coding sequences into this plasmid is possible. Usually a dual construction is chosen where the phagemid vector codes for the pIII protein plus recombinant protein and an antibiotic resistance gene. This phagemid vector can be transformed (see "transformation") into E.Coli. To produce phage with which to conduct experiments a helper phage is used to infect these transformed bacteria. This helper phage contains the full DNA-code for all the phage proteins and allows phage assembly. This results in phage with both wild-type pIII and pIII combined with recombinant protein preserving infectious capability of the pIII while at the same time allowing panning experiments to be performed using the pIII which displays the protein of interest. Another advantage of having both wild-type pIII and fusion protein pIII on the surface is that it allows the selection of recombinant protein with high-affinity to the target protein as opposed to mere high avidity. The helper phage also

contains an antibiotic resistance gene and in this way only those E.Coli survive which have both been infected by helper-phage and carry the phagemid.

Phagemids can be mass-transformed to create a mixture of phage which present hundreds or even millions of different proteins on their surface. This mixture is called a "phage library". Random peptide libraries can be created where phagemids are transformed with randomly created oligonucleotide inserts yielding random proteins on the pIII protein. Another possibility is the creation of natural peptide libraries where the genomes of selected organisms, such as for instance bacteria, are cleaved to the appropriate size and used as inserts in the phagemid for a library where only peptides which occur in these bacteria are expressed.

Production of Fabs using the Pces1 vector

We used the phagemid vector Pces1 which was used in the phage-display method as developed by Hoogenboom[4] to produce Fab-fragments. We did not use this phage-display system for the actual determination of specificity for the Fab fragments thus produced.

The phagemid vector Pces1 contains an ampicillin resistance gene and the DNA encoding the protein III hullprotein (gIII) as shown in figure 12. In front of the gIII region there is a piece of DNA which codes for a protein which becomes fused to the protein III. This is the site where the recombinant protein of interest has to be cloned into the vector. This site and the pIII DNA segment are under the influence of the PlacZ promotor. This promotor is suppressed by glucose and IPTG acts as an irreversible inhibitor. This allows regulation of expression of the fusion protein. Between the DNA segment coding for the recombinant protein and the protein III there is an amber codon. This amber codon stops transcription in about 50% of cases, which means that the protein is also produced without attachment to the pIII. This allows the Pces1 vector also to be used as a general expression system for the recombinant protein, without fusion to the pIII phage protein. We used this possibility to produce our Fab fragment. There is also a His-tag at the end of the recombinant protein which is an aminoacid sequence which allowing binding of the protein using His-binding beads.
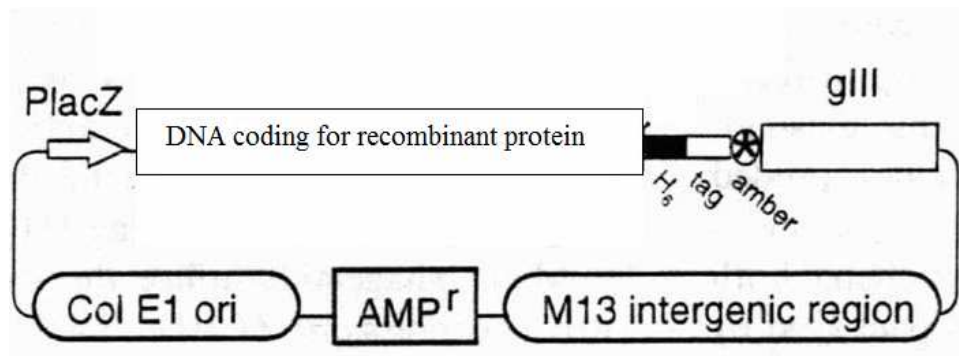
Figure 12:

Schema of Phagemid vector Pces1

For our purpose, the Pces1 vector has been cloned in such a way that following the PlacZ promoter there is a ribosomal binding site (RBS) which is where transcription starts, followed by the VJ-segment of the light chain with either the C_κ or C_λ thereafter, followed by a stop codon which stops translation and ensures the light chain is produced as a separate protein. Thereafter there is another RBS, followed by the DNA coding for the variable and constant domain of the heavy chain. The DNA-coding for the constant domain only codes for the CH1 domain, as this is a Fab-fragment and not a complete antibody. In front of both the light and heavy chain there is a "signal sequence", which causes an aminoacid sequence to be placed before both light and heavy chains which makes sure the produced proteins are excreted into the periplasm. In the periplasm the signal sequence is cleaved off and correct folding of the protein takes place and also disulfide bridges between heavy and light chain are formed. The DNA segment coding for the variable light and heavy chains can be exchanged by cutting the DNA with DNA restriction enzymes, see the picture below. In such a way different Fab fragments can be produced. See below for a more detailed explanation of the DNA restriction sites used.
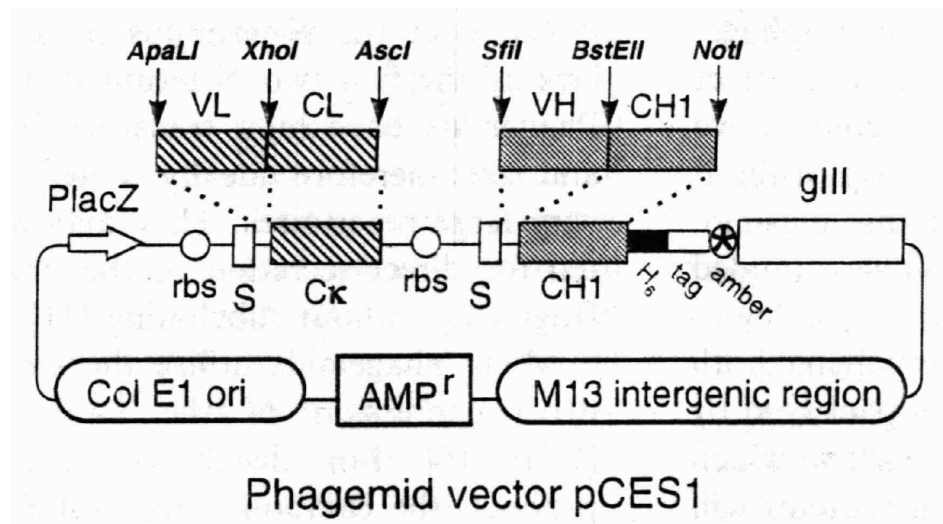
Figure 13:

Schema of Phagemid vector Pces1 with restriction enzyme recognition sites

3. Methods

3.1 Patients

Patient St:

Patients St. was a 48-year old male patient with an acute and aggressive course of generalised WG. About 3 months before the biopsy symptoms started with dyspnoea, recurring headaches and a conjunctivitis which was refractory to therapy. After admission to the hospital the patient was diagnosed histologically with both an active ulcerative conjunctivitis, and a chronic active ulcerative rhinitis. Chest-CT revealed single, partly confluating, intrapulmonary round lesions with a serous pneumothorax. ANCA titer was 1:128 (anti-PR3 36 U/ml). Furthermore, the patient suffered from diabetes insipidus due to a tumor in the pituitary gland, which was diagnosed using an MRI scan, consistent with a pituitary gland granuloma. Even before biopsy an induction therapy according to Fauci was started with high dosed cortisone and cyclophosphamide. In the biopsy of the left lower lobe of the lung the diagnosis of WG was confirmed. Here, there was geographic necrosis with histiocytic walls, single multinucleated giant cells, microabcesses rich in granulocytes and small arteries with transmural lymphoplasmacellular and monocytic inflammatory infiltrates.

Patient Scu:

Patient Scu was a 64-year old female patient with a protracted course of a localised WG. She was ANCA-negative and presented with an aggressive endonasal involvement. Initially she was treated with MTX and therafter with cotrimoxazole for a period of 19 years, after which the biopsy was taken. In the biopsy of the left and right nasal sinuses there was necrosis, inflammatory infiltrates of lymphocytes, plasmacells and granulocytes, as well as epitheloid cells and single giant-cells.

3.2 Overview of methods

As described above we started to work with the DNA coding for the variable domain of the heavy and light chains derived from individual B-cells which were lasered out of granulomatous formations of WG patients. This DNA first has to be sequenced to determine the DNA sequence. To facilitate sequencing we cloned the heavy- and light-chain DNA-fragments into the Topo-vector. Then these DNA-sequences had to be compared to a database of known DNA segments coding for the variable segments of the heavy and light chain to determine if the light chain was either a κ- or λ-chain and also to check if the DNA which was isolated was monoclonal. There is always the danger that the lasering technique which is intended to capture one cell accidentally captures two or even more cells which makes it impossible to determine which light chain belongs to which heavy chain, rendering the sample unusable. Another reason to sequence is to check if the DNA is complete and if not, if certain pieces of DNA need to be added.

Thereafter the appropriate primers had to be devised to perform a PCR reaction leading to the attachment of the appropriate DNA restriction sites to the B-cell DNA, which is only possible if the DNA sequence is known. Thereafter another round of sequencing is necessary to determine if the PCR was successful.

Thereafter the light and heavy chains could be cloned into the Pces1 vector in two steps. When both the heavy and light chains are cloned into the Pces1 vector it is possible to produce the Fab fragments as will be described in detail below.

When we had the Fab-fragments we were able to do two things:

1. Perform integrity testing of the produced Fab fragments using SDS-PAGE and Western blotting.
2. Perform ELISA to test for specificity of the produced Fab fragments.

3.3 DNA and DNA-cloning techniques

DNA

DNA is the molecule which carries the genetic information and it is shown in the following picture.
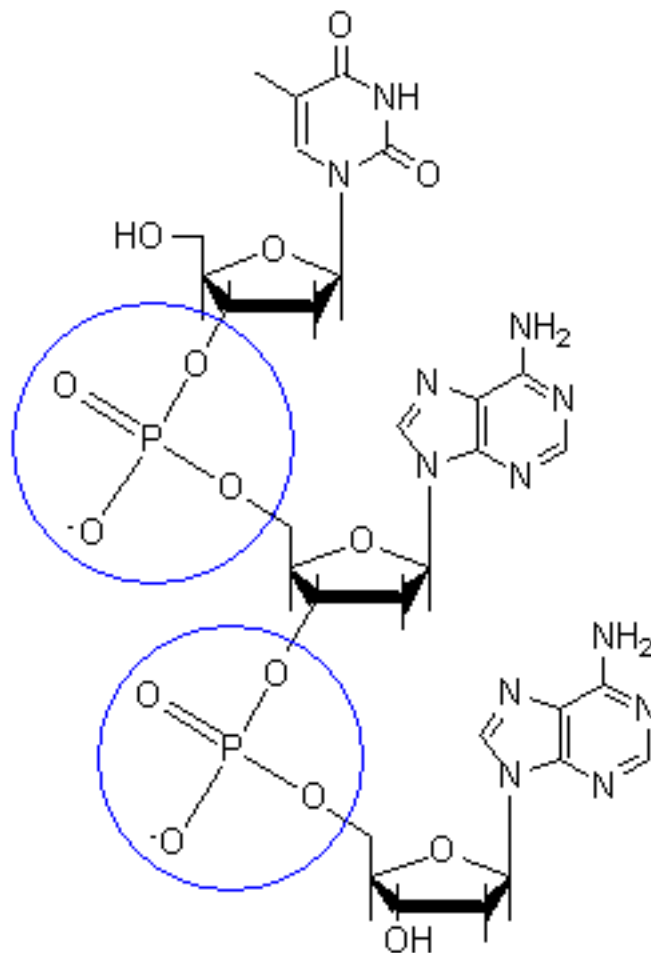
Figure 14:

Molecular structure of DNA

This figure clearly shows that there is a phosphodiester backbone between 2'desoxyribosyl moieties and that this backbone has a polarity. One end has a 5' phosphate terminus and the other end has a 3' phosphate terminus. Attached to this backbone are the purine and pyrimidine bases called adenine (A), thymine (T), cytosine (C) and guanine (G). It is the order of these nucleotides which contains the genetic code. Guanine and cytosine on the one hand and adenine and thymine on the other hand can engage in a specific hydrogen-bond

interaction as shown in the following picture. It should be noted that G-C bonding is stronger then A-T bonding.
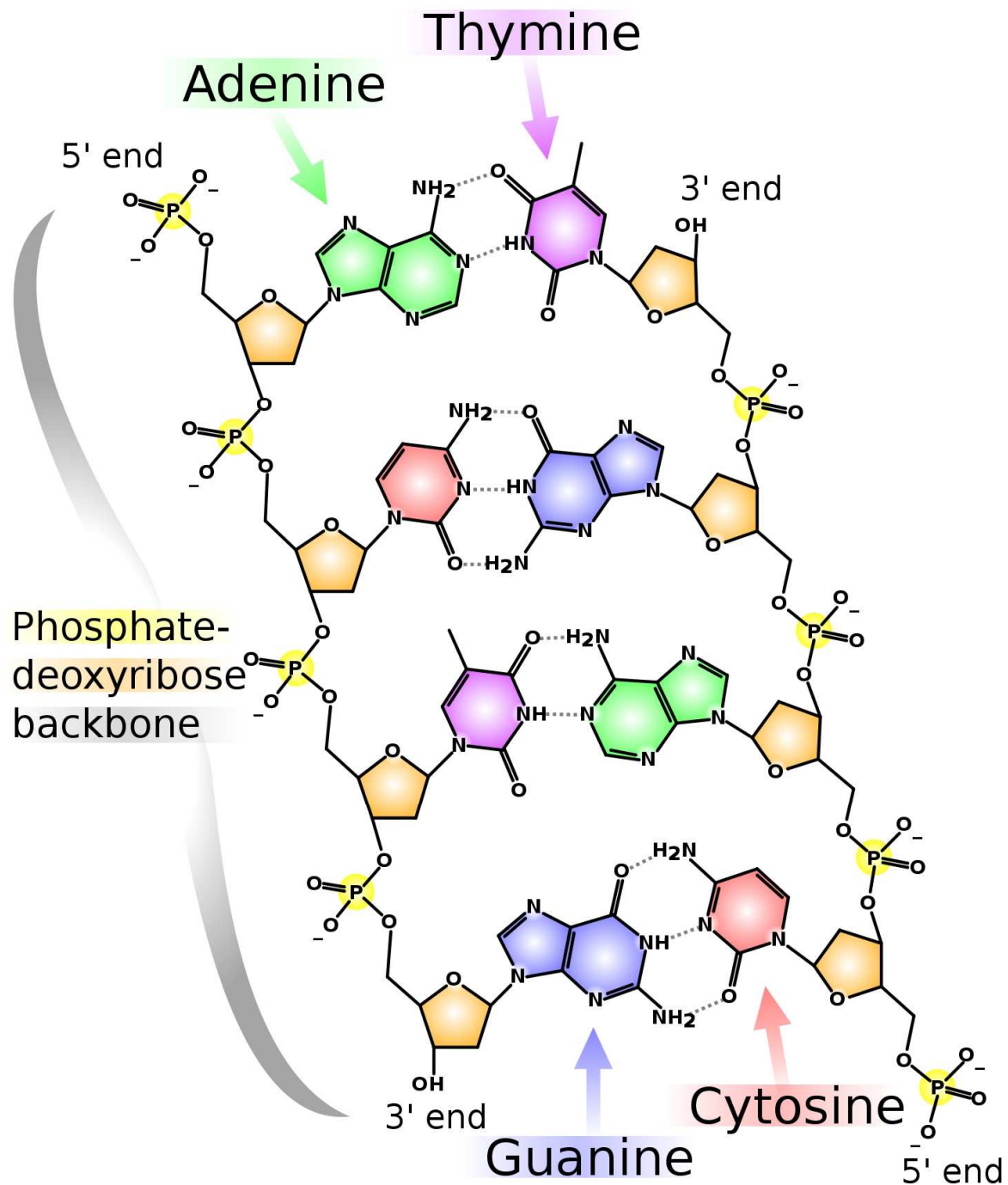
Figure 15:

Base pairing between T&A and C&G is specific

Because base pairing is specific, the nucleotides of two different DNA strands can engage in an interaction whereby the two strands lie opposite eachother and run in an anti-parallel fashion. The two strands form an anti-parallel helix-like structure. This means that one strand runs in the 5' -> 3' direction and the other runs in the 3' -> 5' direction with the nucleotides in the middle, engaging in specific hydrogen-bond interaction, where each C on one strand couples with a G on the other strand and each T on one strand couples with an A on the other strand as shown in the following picture, which shows the helix formed by the DNA-molecules.
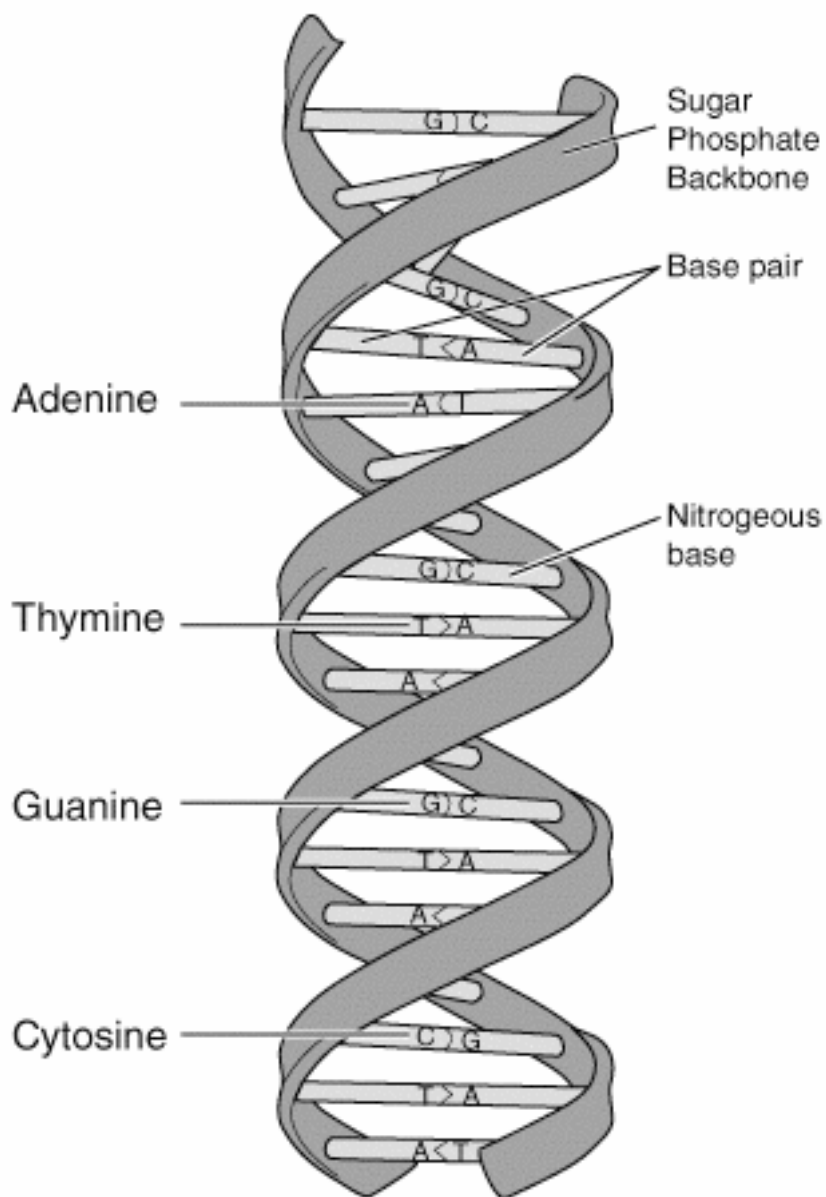
Figure 16:

Double-helix structure of two DNA strands.

The DNA-sequence of two DNA strands can be represented schematically as follows:

5'-GACTACTA-3'
3'-CTGATGAT-5'

The upper strand is called the 'sense' or 'coding' strand because it is used during translation to determine the amino-acid sequence of a protein. The other strand is called the anti-sense or anti-coding strand. Both strands are *complementary* to eachother because of the specific coupling of the two pairs of nucleotides described above.

Restriction enzymes

Restriction enzymes are enzymes which cut double-stranded DNA at specific sites. Each enzyme recognizes a specific nucleotide sequence and then cuts the DNA in a specific way. There are restriction enzymes which make 'blunt' ends and there are restriction enzymes which make 'sticky' ends. An example of a restriction enzyme which makes sticky ends is EcoRI.

The EcoRI restriction enzyme recognizes the GAATTC nucleotide sequence. The nucleotide sequence before or after this sequence is irrelevant and is therefore shown as x's in the example below.

Nucleotide sequence before cutting of DNA with EcoRI

xxxxxxxxxxxxxxxxxxxxxxGAATTCxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxCTTAAGxxxxxxxxxxxxx

Nucleotide sequence after cutting of DNA with EcoRI

xxxxxxxxxxxxxxxxxxxxxxG   AATTCxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxCTTAA   Gxxxxxxxxxxxxx

As can be recognized from the illustration above the restriction enzyme has cut the DNA and 'sticky' ends have been created. These ends are called 'sticky' because they have a DNA 'overhang', which means at their end they are partly single-stranded. If the sticky ends are complementary to eachother they can form one DNA-molecule again if the phosphodiester bonds are once again formed. There are also restriction enzymes which create non-sticky-

ends. That means both ends are not complementary to eachother because the overhangs do not match.

The SmaI restriction enzyme makes 'blunt' ends as shown here.

Nucleotide sequence before cutting of DNA with SmaI

xxxxxxxxxxxxxxxxxxxxxxCCCGGGxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxGGGCCCxxxxxxxxxxxxx

Nucleotide sequence after cutting of DNA with SmaI

xxxxxxxxxxxxxxxxxxxxxxxCCC   GGGxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxxGGG   CCCxxxxxxxxxxxxx

We used restriction enzymes from Fermentas (Burlington, Canada). Each enzyme recognizes its restriction enzyme recognition site and each enzyme requires a certain buffer where it functions optimally. Fermentas company has three different buffers which are used with their enzymes. A typical reaction setup is the following.

Plasmid DNA or linear DNA to be digested	4 µl	(which represents for instance 2,5 µg DNA)
10x Puff (yellow, green, or red)	2 µl	
ApaLI (restriction enzyme)	1 µl	
XhoI (restriction enzyme)	1 µl	
H_2O	12 µl	
	--	
	20 µl	

This reaction mix was then incubated at the temperature where these enzymes operate best for one hour or overnight. Some enzymes can also be deactivated by heating at a certain temperature to avoid overdigestion, which is when the enzymes start to cut the DNA randomly when all the restriction enzyme sites have already been digested.

Most enzymes have 10 units/µl. One unit is defined as the amount of enzyme required to digest 1 µg of lambda DNA in 1 hour at the appropriate temperature in 50 µl of assay buffer.

As a rule of thumb 3-4 units/µg DNA to be digested can be used to digest over a time period of at least 2 hours. In the example above therefore 10 units were used (1 µl = 10 units)

Ligation

A ligase is an enzyme which joins DNA-molecules with *compatible*, "sticky", ends to eachother by forming the phosphodiester bonds which are present in the DNA backbone.

A ligase will join all sites with compatible sticky ends to form a contiguous DNA molecule. As long as the ends are sticky, the ligase will link the two ends together. The two ends can be from different DNA-molecules or they can be from the same DNA molecule. The latter is the case if both ends of a linear DNA molecule have compatible sticky ends, thereby forming a circular DNA molecule. In this way it is possible to insert a piece of DNA into a plasmid, which is a circular piece of DNA (see below). First, the plasmid is cut with a restriction enzyme yielding a linearized DNA with sticky ends. Secondly, the DNA-fragment which has to be inserted is also cut with the same restriction enzyme. Therefore the sticky ends of both the linearized plasmid and the DNA insert are compatible and 'annealing' can take place. This means that the nucleotides at the sticky end form the complementary hydrogen bonds. Then the ligase forms the phosphodiesterbonds in the backbone, thereby forming one circular DNA molecule as shown below.
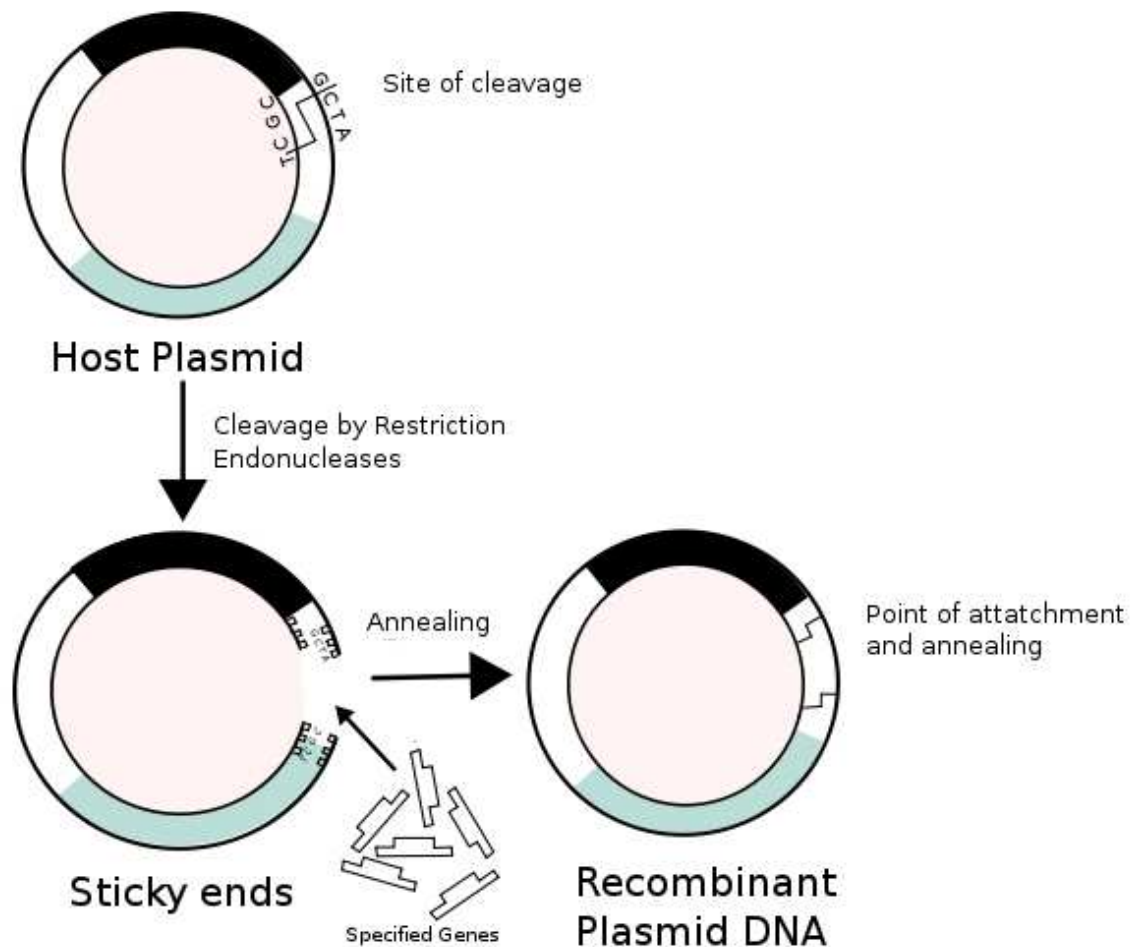
Fig 17:
Insertion of a DNA segment in a plasmid

The optimal *molar* ratio between insert and vector is 3:1. As the insert in most cases is much smaller than the vector, the weight of DNA which is to be used can have a very different ratio. We used T4 DNA Ligase, which is a bacterial ligation enzyme sold by Roche Diagnostics (Risch, Switzerland). This enzyme is provided with a 10x ligation buffer. The following setup is typical for ligation of an insert into a vector.

Below is an example of a ligation of a 1200 bp insert into a vector which is about 3600 bp. The ideal molar ratio is 3:1. Because the insert is three times smaller than the vector equimolarity is achieved when using equal amounts of DNA weight.

digested Vector	2 µl
digested Insert	2 µl
10x Ligation Buffer	2 µl
T4 Dna Ligase	0,5 µl
H$_2$O	13,5 µl
	--
	20 µl

Cloning of heavy and light chain DNA into Topo Vector

A plasmid is a circular piece of double-stranded DNA which exists in the bacterial cytoplasm and uses the enzymes within this cytoplasm for DNA replication and protein synthesis. The plasmids used in DNA recombination technology always contain a number of elements. There is the origin of replication, allowing the plasmid to be replicated and to survive as the bacterial cell divides. It contains an antibiotic resistance gene allowing selection of those bacteria who carry the plasmid within them. It also contains a "multiple cloning site", which is a part of the DNA sequence of the plasmid where multiple restriction enzyme recognition sequences are located. This allows cloning of DNA fragments into the vector as described under "ligase". A plasmid can also contain other protein-coding sequences, which gives the bacteria which carry the plasmid in their cytoplasm certain additional characteristics, depending on the proteins which are synthesised from transcription of the plasmid DNA. Because we had to sequence relatively short DNA fragments we chose to clone our DNA into a plasmid vector first. This allows complete sequencing of even short DNA fragments, without loss. The technical reasons for this are detailed in "sequencing". The Zero Blunt TOPO PCR cloning kit from Invitrogen was acquired to clone the heavy- and light-chain fragments into the pCR-Blunt II-TOPO vector which is shown in the following figure.
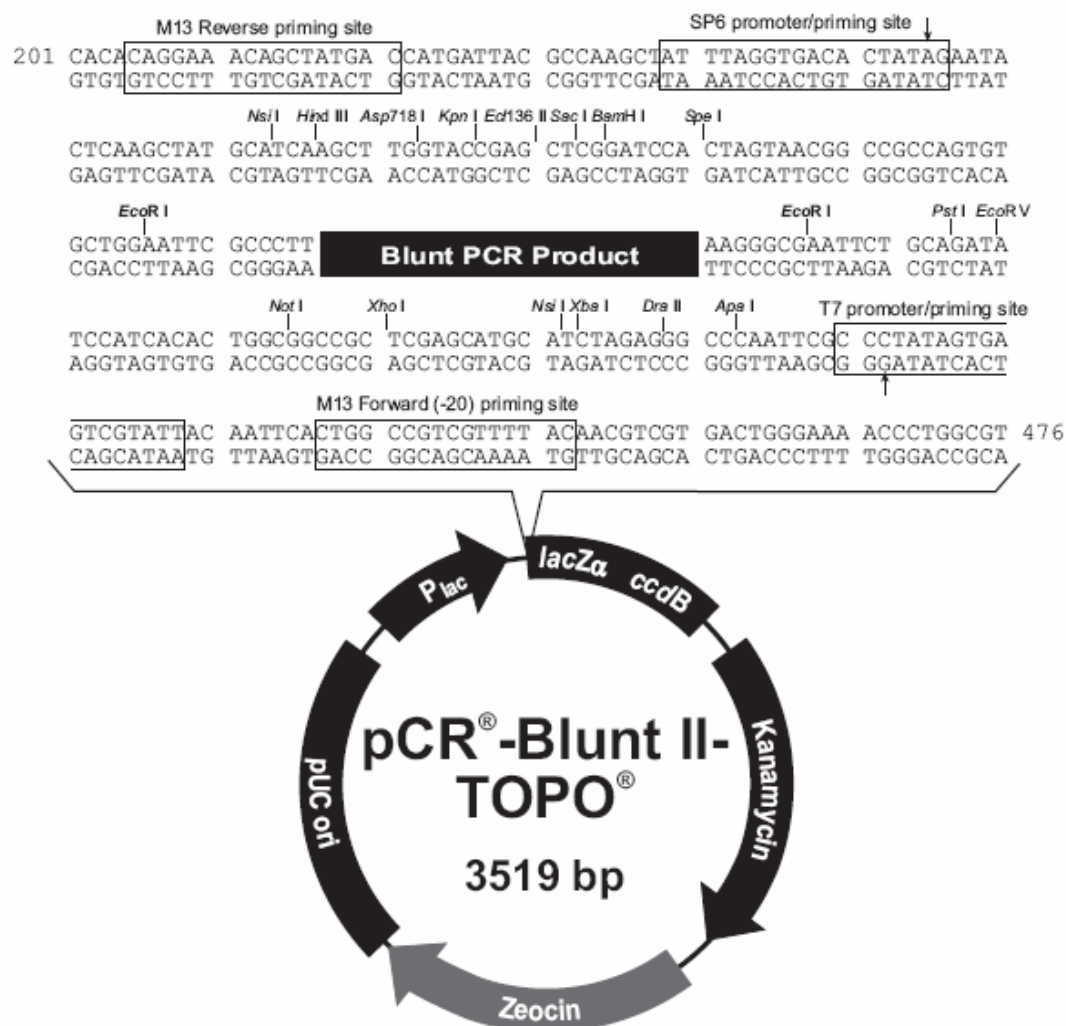
Figure 18:

Schema of pCR-Blunt II-TOPO vector[11],

Copyright of Invitrogen (Karlsruhe, Germany)

The vector is a circular piece of DNA of 3519 base pairs in total and the multiple-cloning site is shown in the figure above. This vector contains both a kanamycin and zeocin antibiotic resistance gene. In our experiments we used Kanamycin as a selecting agent. The vector we used is special because in the middle of the multiple cloning site there is a site where DNA can be cloned into the vector without the use of a restriction enzyme. Therefore, blunt DNA-segments can be cloned into this vector without the need for compatible sticky ends. Therefore this vector can be used for cloning virtually any piece of DNA as long as it has blunt ends. The vector comes as a linearized piece of DNA with a protein called "Vaccinia virus DNA topoisomerase" covalently bound to each end as shown in the following figure or cartoon.
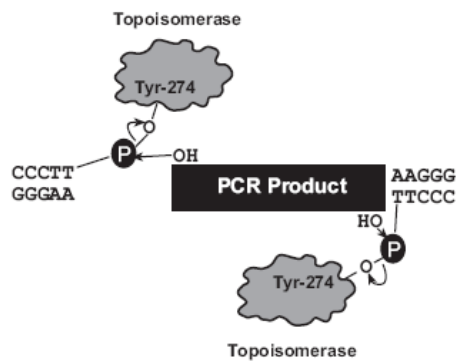
Figure 19[11]:

Cloning into the TOPO vector,

copyright of Invitrogen (Karlsruhe, Germany)

As shown in figure 19, a double-stranded piece of DNA with blunt ends can covalently attach to the DNA-strand of the linearized plasmid with both its 5' and 3' hydroxyl group, releasing the topoisomerase leading to the insertion of the DNA fragment into the linearized plasmid, once again forming a normal, circular plasmid.

There is also a Topo Vector available which allows the cloning of DNA fragments with TA-sticky ends, that is the end of the vector has a T overhang. We used this vector to clone our light- and heavy chain DNA fragments because in the two rounds of PCR, as described in "preliminary work" an enzyme which makes these TA-overhangs was used.

The following protocol was used to clone our PCR-products into the vector and is called the "TOPO cloning reaction".

TOPO cloning reaction, also see "Kit contents, media and solutions"

PCR Product	2 µl
Salt Solution	1 µl
Water	2 µl
pCR II-Blunt-TOPO	1 µl

This is basically a mix of the DNA insert and the vector with the topoisomerase at its ends in a salt solution to facilitate the reaction described above. This mix was incubated for 5 minutes at room temperature so the reaction could take place. After this we proceeded with "transformation" of the plasmid into DH5-α bacteria for reasons discussed below.

Transformation and production of competent DH5-α bacteria.

Transformation is the process whereby DNA is introduced into bacteria. We used the "chemical" way of transformation. There is also an "electroporation method" where the DNA is transferred across the cell membrane by the application of a high-voltage shock. To guarantee a high efficiency of DNA take-up into the bacterial cell, DH5-alfa cells were treated using a protocol to make them "competent", which means they are treated so their cell membrane takes up DNA more efficiently.

We used the following protocol to produce competent DH5-α bacteria:

DH5-α bacteria are grown overnight at 37 °C on an agar plate with SOC-medium. Ten to twelf 2-3 mm large single colonies were picked and used to inoculate 250 mL of SOC-medium and were incubated shaking at 18-22 °C in a glass 2 liter container until the OD at 600 nm was about 0.6, which took about 20-24 hours. The container was put on ice for 10 minutes. Then the cells were transferred to 400 ml Beckman containers and centrifuged at 3000 rpm at 4 °C for 10 minutes. The supernatant was discarded and resuspended in 130 ml of TB solution and incubated on ice for another 10 minutes. After that the bacteria were once again centrifuged at 3000 rpm at 4 °C for 10 minutes and again resuspended in 25 ml TB-solution. Under a light swinging motion 1.9 ml DMSO was added, which means the DMSO-concentration was 7%. Thereafter the bacteria were incubated another 10 minutes on ice and aliquoted into 1.8 ml cryotubes. These tubes were then shock-frozen in liquid nitrogen after which the solution containing the bacteria was stored at -80 °C for future use.

The principle of inducing competency in these cells is based on the high chloride concentration in the TB solution. The chloride in this solution enters the cells and causes osmotic swelling which stretches the cell membrane. The exact mechanism for induction of competency however is unknown.

After the TOPO cloning reaction the newly formed circular plasmid was transformed into competent cells using the following protocol.

1. The solution which contains the competent DH5-α bacteria stored at -80 °C was thawed on ice
2. For each topo cloning reaction 100 µl of this solution were aliquoted
3. 4 µl of the topo cloning reaction mix (containing the plasmid) was added to this 100 µl
4. This mixture was incubated on ice for 30 minutes

5. After this, the mixture was heat-shocked for 45 seconds at 42 °C after which the solution was once again placed on ice

6. 400 ml SOC medium was added

7. This mixture was incubated on a shaker at 37 ° C for one hour

8. The bacteria were plated on an LB-agar-Kanamycin plate and incubated overnight at 37°C.

The bacteria should be treated very carefully throughout, avoiding shocks, as competent bacteria are very fragile. Therefore the bacteria are handled on ice. After mixing the TOPO cloning reaction mix with the competent bacteria, the DNA will be taken up and the heat-shock adds further to transformation efficiency. After the heat shock the bacteria will contain the plasmid and transcription can take place. In this way the antibiotic resistance will develop. The SOC medium is added as nutrient for the cells and the one hour incubation time is the time in which the antibiotic resistance develops, after which the bacteria are plated and only the bacteria which have been successfully transformed survive. Because the number of bacteria is much larger than the number of DNA molecules, each bacterium will contain only one plasmid and most bacteria will not take up any plasmid and gain no antibiotic resistance.

The next day colonies can be picked and can be used to inoculate a LB medium which yields a homogeneous population of bacteria within this medium. All these bacteria are derived from one bacterium and all these bacteria carry within them the same plasmid with the same DNA insert.

This procedure has a number of advantages. As described above this method yields bacterial cultures which are monoclonal. This is an advantage when cloning a PCR product into the TOPO vector because after the cloning reaction there is a mixture of plasmids with different inserts. That is because the PCR product contains a lot of different DNA fragments: there are the primer DNA fragments, and there are various PCR reaction products, the majority of which will be the intended reaction product, but there are also a lot of DNA products in the mixture which are a result of non-specific binding of the primers to the template DNA yielding non-sense DNA. The method of transformation as described above allows the isolation of plasmid with only one distinct insert, as opposed to a mixture of plasmids with different inserts, as it exists right after the TOPO cloning reaction. The second advantage is that in this way large quantities of bacteria carrying the plasmid are produced, as the inoculated medium can be of any chosen volume. This allows large quantities of DNA to be

isolated carrying the PCR product of interest. Therefore this procedure represents a very effective way for the isolation of very pure DNA in large quantities.

As a sidenote, bacterial competency should always be checked before doing the transformation reaction. Therefore 1 µg of DNA has to be used to transform the bacteria using the abovementioned protocol. Because 1 µg is a lot of DNA it is wise to dilute the transformed cells before plating, for instance 10,000x. Then the number of colonies has to be counted. From this the number of colonies per µg DNA used in transformation can be calculated for a particular batch of bacteria. The unit of competency is colony forming units/µg DNA.

To isolate the plasmids from the monoclonal bacteria which are present in the overnight-culture we use the TENS-protocol.

Preparation of DNA in "mini"-quantities using the TENS protocol

This is a protocol which can be used to prepare plasmid DNA from bacterial cultures, yielding DNA quantities with a maximum of about 20 µg.

1. 1.5 ml of the bacterial culture is centrifuged full-speed for 30 seconds
2. Supernatant is discarded
3. Pellet is resuspended in 100 µl buffer S1 + RNase
4. 300 µl TENS-buffer is added
5. 150 µl 3M NaAc (pH 5.2) is added
6. The suspension is shaken
7. Centrifugation full-speed for 10-20 minutes
8. Supernatant is transferred to new tube
9. 1 ml -20°C 100% alcohol is added, vortex
10. Mixture is centrifuged full-speed for 10-20 minutes
11. Supernatant is discarded
12. Pellet is resuspended in 1 ml 70% alcohol
13. Mixture is centrifuged full-speed for 10-20 minutes
14. Supernatant is discarded
15. Pellet is allowed to dry
16. Pellet is resuspended in 25-50 µl of water

Usually this will yield a DNA concentration of about 100-250 µg/µl when using a high-copy plasmid such as the TOPO vector.

The DNA which has been obtained in this way must thereafter be cleaned of RNA and other bacterial debris, see "DNA and Gel Band Purification".

So, after cloning each light- and heavy chain PCR product into the TOPO vector we transformed the plasmid into DH5-α bacteria and plated the bacteria on LB-agar-Kanamycin plates and picked colonies. For each transformation we picked 10 colonies and sent these to a commercial laboratory for sequencing.

DNA Gel Electrophoresis

It is possible to separate a mixture of DNA molecules based on their size with an agarose gel electrophoresis. The DNA is mixed on the gel and an electrical field is applied which causes the DNA molecules to move through the gel. Larger molecules will move slower then small ones. After some time the separation of the DNA molecules can be visualised by illumination with a UV light. The DNA will be visualized because ethidiumbromide is present in the gel. The combination of ethidiumbromide and DNA is fluorescent under UV-illumination. The illumination pattern can then be compared to a "ladder" which is a DNA mixture with DNA molecules of known size.

First, the agarose gel was prepared according to the following recipe for a 1.5 % agarose gel

1. Prepare TAE from a stock solution of 50x TAE
2. Add 1.5 gram of agarose to 100 ml of this stock-solution
3. Cook in a microwave until the agarose is dissolved
4. Let the solution cool off, and add water which has evaporated by cooking
5. Add ethidiumbromid to a final concentration of 0,5 µg/ml
6. Poor this solution into a container which forms the gel including the holes for loading the DNA and remove air bubbles
7. Let it cool off until the gel is solid

After the gel is ready the DNA is loaded into the gel using the loading holes, but before loading the DNA is mixed with loading dye. The gel is also loaded with a 'ladder' to compare DNA size. The "ladder" is a mixture of DNA fragments with known size so that it can be used

as a reference to determine the size of an unknown DNA fragment in a gel. We used the "Generuler" from Fermentas. Then a voltage of about 100 V is applied for about 30-45 minutes. An example illustrating the principle of a DNA ladder is shown in Figure 20.
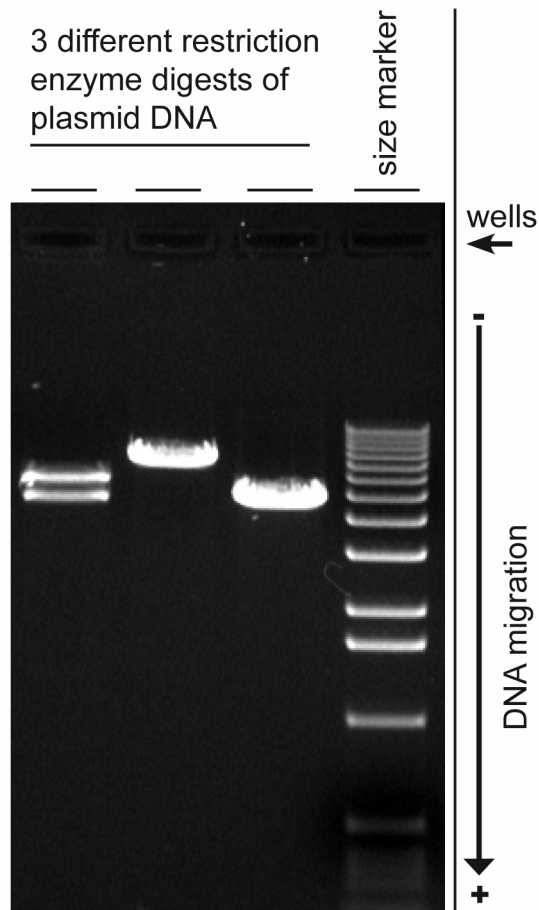
Figure 20:

DNA ladder used in gel electroforesis

After the gel electrophoresis has been run, the mixture of DNA was separated and the appropriate bands of DNA can be cut out of the gel using a scalpel. Thereafter the DNA has to be prepared from the gel.

DNA and Gel Band Purification

For purifying DNA from solution or an agarose gel we used the "GFX PCR DNA and Gel Band Purification Kit" from Amersham Biosciences (Amersham, UK). This kit can be used to purify DNA from a solution which also contains protein (for instance cellular debris from TENS-preparation), salts, primers (after a PCR), restriction fragments (after restriction enzyme digestion of DNA) or to isolate DNA from a TAE gel.

The principle is as follows: the gel or solution is incubated with an agent which denatures protein, dissolves agarose and promotes binding of DNA to a glass fiber matrix. The fiber matrix is then washed with a washing solution and thereafter the DNA is eluted from the matrix using water. All these steps involve centrifugation between steps. DNA fragments which are captured are from 100 bp to 48 kbp.

Polymerase chain reaction

The polymerase chain reaction is a method with which a target sequence of DNA can be multiplied. Normal DNA replication, which occurs when the cell divides and two identical copies of the double-stranded DNA molecule have to be generated is performed by an enzyme called DNA polymerase. DNA replication takes place when the two strands dissociate from eachother under the influence of certain enzymes, thereafter each strand is used as a template to create the opposing strand again, thereby generating identical copies. As the DNA polymerase moves along the DNA molecule two new molecules are created. This principle is shown in the following two pictures. Note that DNA replication naturally takes place in a discontinous fashion on one of the two strands which are being replicated, since the DNA polymerase can only operate in the 5' to 3' direction. DNA replication always needs a certain "primer", this is a short stretch of RNA which is complementary to part of the DNA strand. The place where the primer binds to the DNA template is where the DNA-polymerase can start its work. There are many places where these RNA primers bind and therefore many stretches where a piece of DNA has been replicated, such a stretch is called an Okazaki-fragment, see figure 22.
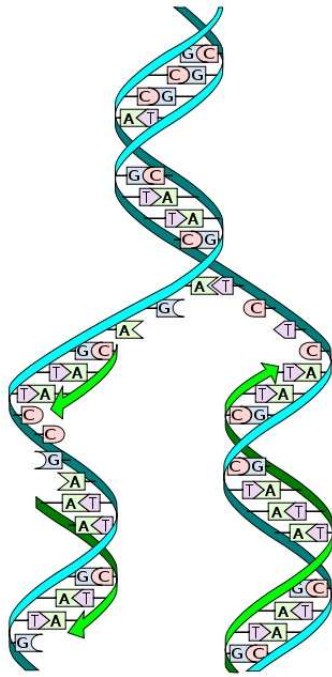
Figure 21:

Illustration of DNA-replication
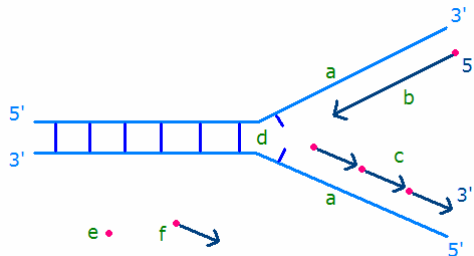
Figure 22:

Okazaki fragment,

e=primer, f=Okazaki-fragment

The DNA polymerase molecule can be used in vitro to multiply DNA molecules too. Let's assume we have a piece of DNA which is a couple of thousand basepairs long. Within this DNA molecule there is a certain segment which is important to us and we want this segment to be multiplied. We can then make a certain reaction mix containing this DNA molecule, the DNA polymerase, a mixture of nucleotides (G,C,T,A) and two primers in water with appropriate pH and salt concentrations. The primers are necessary as starting point for DNA replication as we saw above. In vitro, however, the primer is not a piece of RNA, but a short piece of DNA. The first primer is constructed in such a way that it is complementary to the

sense strand at the start of the DNA segment which is to be amplified. The second primer is complementary to the anti-sensestrand at the end of the DNA segment which is to be amplified. By putting this mixture through an automated series of temperature changes it is possible to repeat DNA replication of the DNA segment between the two primers.

The reaction mixture is put through the following temperature steps. First, the temperature is raised to 95°C to cause the two strands of the DNA molecule to dissociate ("denaturation"), allowing the primer to attach to the single DNA strands at a temperature which is dependent upon the length and nucleotide composition of the primers, and is generally about 50-70°C. This process is called "annealing". Thereafter, DNA replication can take place at a temperature of 72°C, which means that the DNA polymerase makes a double-stranded molecule again by complementing each nucleotide on the template DNA strand using the nucleotides present in the reaction mixture. This temperature is of course much higher than the temperature at which normal DNA replication takes place. This can be explained by the fact that the DNA-polymerase which is used in vitro is a bacterial DNA polymerase which can withstand much higher temperatures than human DNA polymerase. This is necessary because denaturation temperatures are very high and would inactivate the human DNA polymerase. This cycle can be repeated many times. Each time only the DNA segment between the primers is replicated and in such a way the DNA is replicated 10^9 times after 30 cycles. The principle is illustrated in the following picture.
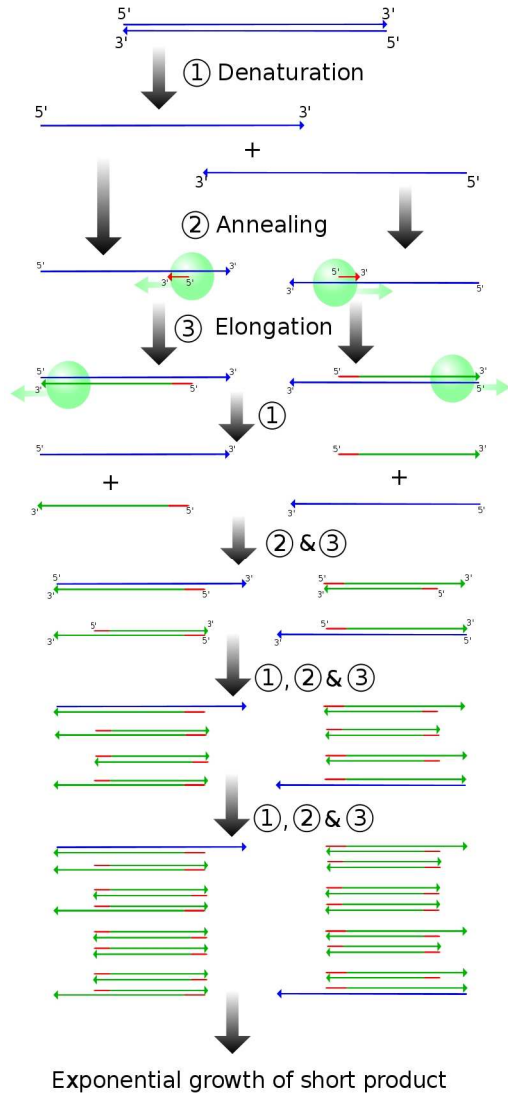
Figure 23:

Illustration of the principle of the polymerase chain reaction

As indicated above, the annealing temperature to be chosen depends upon the length of the primer and the nucleotide content thereof. As a rule of thumb the following formula can be used to calculate annealing temperature (T_m).

$$T_m = 2°C \ (A+T) + 4°C \ (G+C)$$

Where A,T,G,C represent the number of these nucleotides in the primer.

When designing primers it is important to have a G or C at the end of the primer because the bonding between the G and C nucleotides is stronger then the bonding between T and A, in

this way the chance for proper annealing is increased. When the nucleotide sequence of a certain DNA molecule is known, usually only the sense strand is shown. For instance:

5'- AGGCTTGGTGCCTCCGCCGAA<u>CACCTCGTGCTGCTATCATAAGACTGACAG TAGTAGTCAGCCTCGTCCT</u>CAGTCTTCAGTCCAGAGA-**3'**

If we want to amplify the underlined DNA segment we should make two primers as follows. The first primer, which designates the start of the DNA segment to be amplified, is called the 5' primer. This primer attaches the anti-sense strand. Therefore, it has to be complementary to the anti-sense strand and therefore the 5' primer is equal to the start of the DNA segment to be amplified:

<u>5'- Primer</u>
5'-<u>CACCTCGTGCTGCTA</u>-3'

The primer which designates the end of the DNA segment to be amplified is called the 3' primer. This primer attaches to the sense strand. If we take the end of the DNA segment to be amplified from the sequence above and draw in the anti-sense strand it would look like this:

```
5'TCAGCCTCGTCCT-3'
3'AGTCGGAGCAGGA-5'
```

From this picture it is clear that if we are to make a primer which attaches to the sense strand we should take the *reverse complement* of the sense-strand which is:

<u>3'- Primer</u>
```
5'AGGACGAGGCTGA-3'
```

Primers are always synthesized in the 5' -> 3' direction.

We ordered our primers from MWG Biotech (Ebersberg, Germany).

Using the formula which was shown above, we can calculate the annealing temperature for our primers. When we use longer primers the annealing temperature will be higher. The

advantage of using longer primers is that the specificity increases. With increasing primer length the chance of primer-binding to DNA segments, which are similar to the DNA segment to be amplified, decreases. In this way PCR can be used to amplify a segment of DNA which is present in a mixture of DNA molecules or to amplify one small part of the whole genome, all because primer binding is highly specific. In the single cells which were isolated from granulomatous lesions of WG patients, the heavy- and light-chain coding segments are only 350 basepairs long and the rest of the entire humane genome is also present. After the PCR reaction, however, most of the DNA in the reaction mix is the DNA which codes for the heavy- or light chains of the antibody because this DNA segment has been amplified tremendously.

Another very important application of PCR is the possibility to add nucleotides to the DNA segment which is to be amplified. We did this by adding the nucleotides in front of the primer sequence we designed. We used this to add the restriction enzyme recognition sites in front of the DNA segments we work with, so we could use these sites to make "sticky" ends, so we could clone the DNA-segment into for instance the Pces1 vector. Annealing temperature was calculated only on the basis of the formula given above.

If we wanted to add the GAATTC (EcoRI) restriction enzyme recognition sequence at both ends of the DNA segment in the example we presented above we would change the sequence of our 5' and 3' in the following way:

5'- Primer

5'-*GAATTC* <u>CACCTCGTGCTGCTA</u>-3'

3'- Primer

5'*GAATTC* <u>AGGACGAGGCTGA</u>-3'

This would result in a DNA fragment after the PCR reaction with the following sequence:

5'- *GAATTC* <u>CACCTCGTGCTGCTATCATAAGACTGACAGTAGTAGTCAGCCTCGTC</u> <u>CT</u> *GAATTC*-3'

The primer will attach, even though during the first cycle the first few nucleotides (GAATTC) of the primer have no match in the DNA template. This is no problem as long as the number

of added nucleotides is not too big. After annealing the DNA replication will start at the beginning of the primer, thereby incorporating the added nucleotides into the DNA molecule. In subsequent rounds, the primer will attach 100% correctly to the DNA-template since the added nucleotides will now be available in the DNA template.

It is also possible to use a mixture of primers, if large numbers of nucleotides have to be added. More about this in "results".

We used Proofstart ™ DNA polymerase from Qiagen® 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	
	--		
	50	µl	

The following PCR program is used:

Step 1 Activation: 5 min 95 Grad

Step 2 Denaturation: 1 min 94 Grad

Step 3 Annealing: 1 min 50-72 Grad

Step 3 Extension   1 min 72 Grad

Step 4 Repeat   goto 2 30 times

Step 6 72 Grad 7 Minuten

Step 7 Hold at 4°C forever

Sequencing

Sequencing is a method to determine the nucleotide sequence of DNA. We sent our DNA-samples to a laboratory which used the Sanger method of sequencing. In this method four PCR reactions are set up using a single primer which binds to the DNA-segment of the DNA molecule where sequencing should start is included. Within the reaction mix there are the four nucleotides which are used for DNA synthesis as in all PCR reactions. In each of the four reactions one type of nucleotide is also present as a dideoxynucleotide. When this nucleotide is used for DNA-synthesis it leads to termination, because no further nucleotides can be attached to the dideoxnucleotide. Because the concentration of the dideoxynucleotide is only 0.02 % of the concentration of the normal nucleotides the termination events, for statistical reasons, generally take place within the first 200-500 basepairs after the start of DNA synthesis, which is at the site where the primer binds. Therefore, after the PCR there is a mixture of DNA-fragments with a length of up to 500 basepairs. Either the primer or one of the normal nucleotides can be marked with a fluorescent or radioactive substance. This makes it possible to determine the length of the different DNA fragments by applying it to an electrophoresis gel which separates the DNA fragments based on size. This enables determination of the nucleotide sequence of the original template. When a termination event takes place it means that at that position a certain dideoxynucleotide was built in which identifies the complementary nucleotide on the template-DNA strand. For each of the four reactions a number of DNA fragment sizes will be found. For each reaction, the sizes of these DNA fragments indicate where this nucleotide can be found in the DNA sequence, relative to the the primer-annealing site. In this way the identity of each nucleotide of the template DNA strand can be identified by combining the results of the four reactions. This is shown in the following figure.
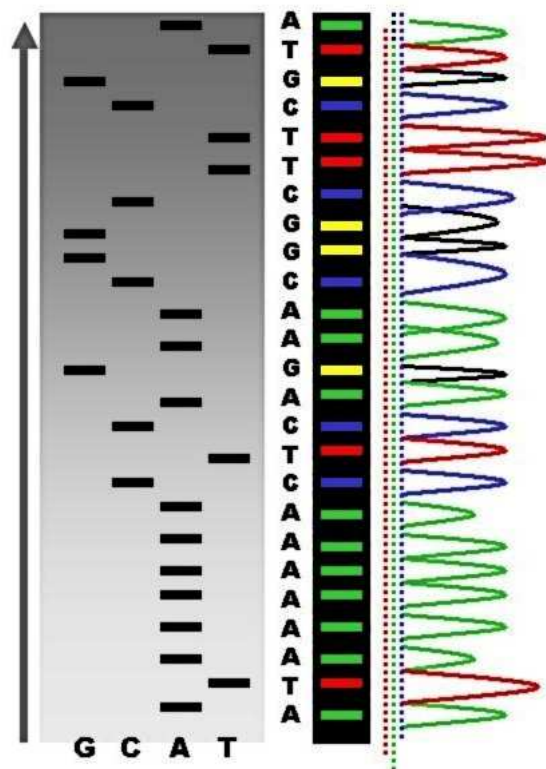
Figure 24:

Principle of sequencing according to Sanger

When using the TOPO vector, we used the M13 Reverse primer, which attaches to the M13 reverse priming site as a starting point for sequencing. With this primer, the insert in the TOPO vector is always sequenced.

3.4 Cloning of variable chain coding DNA segments into the Pces1 vector

Using the techniques described above it is possible to prepare the variable chain segments in such a way that they have the restriction enzyme recognition sequences forming sticky ends. The appropriate restriction enzyme recognition sequences are also available in the Pces1 vector and after a restriction enzyme digest the DNA insert will have compatible sticky ends so it can be ligated into the Pces1 vector. This is depicted in the following picture.
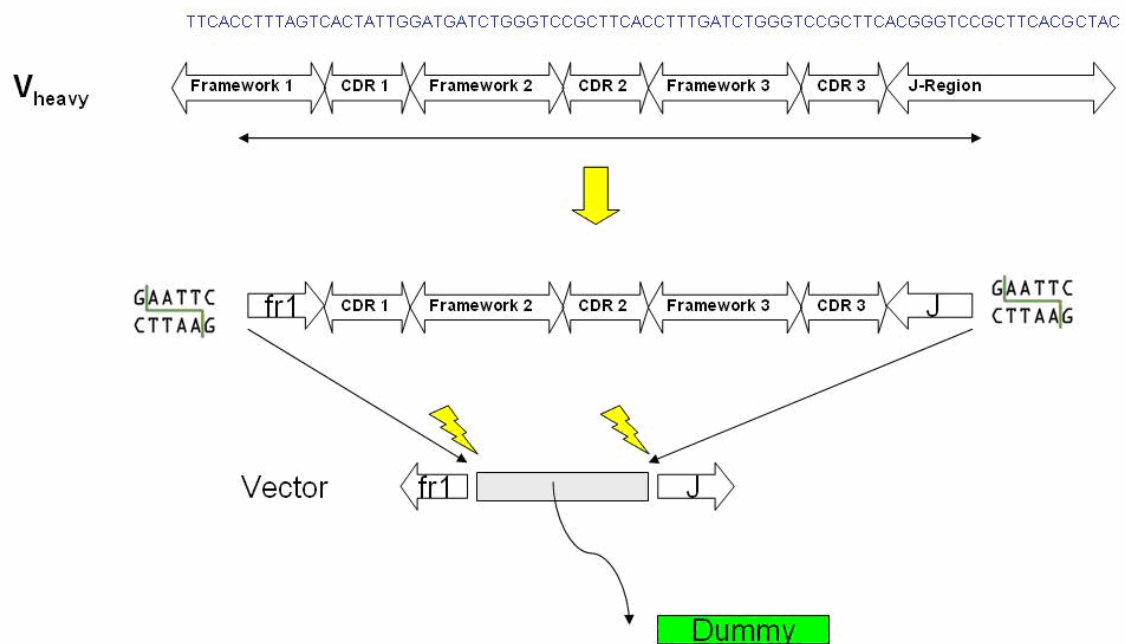
Figure 25:

Schematic picture of the cloning reaction

The picture above shows schematically that a variable chain coding DNA segment was put through a PCR to add the restriction enzyme recognition sites to its ends. After a digest of this insert the DNA has sticky ends. The vector is also digested, which frees a "dummy" piece of DNA as a restriction fragment. The vector and the dummy insert can be separated from eachother on an agarose electrophoresis gel and the digested Pces1-vector at this point also has sticky ends and can then be cut out of the gel to be incubated with the insert in a ligation reaction. After that transformation and DNA preparation can take place.

For a better understanding of the exact cloning reactions we performed here, again, is the figure showing the Pces1 vector.
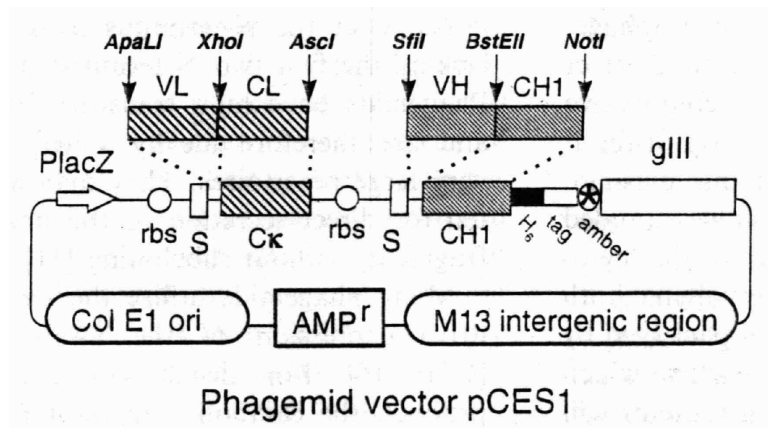
Figure 26:

Pces1 vector, S = signal sequence, Cκ = constant domain of K light-chain,

CH1 = constant domain of heavy chain

The exact DNA sequence of the Pces1 restrictiction enzyme recognition sequences is shown below. The light chain was inserted into the vector using the ApaLI restriction enzyme at the 5' end and the XhoI restriction enzyme at 3' end. For the heavy chain the SfiI and BsteII restriction enzymes were used to clone the insert into the vector.

ApaLI
```
5'-G^T G C A C-3'
3'-C A C G T^G-5'
```

XhoI
```
5'-C^T C G A G-3'
3'-G A G C T^C-5'
```

SfiI
```
5'-G G C C N N N N^N G G C C-3'
3'-C C G G N^N N N N C C G G-5'
```

BsteII
```
5'-G^G T N A C C-3'
3'-C C A N T G^G-5'
```

These restriction enzyme sites can be found in the vector at appropriate locations as indicated below.

Part of Pces1 vector DNA sequence

--

Signal sequence

GTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATTCTCACA

ApaLI

<mark>GTGCAC</mark> AA

Variable chain (κ)

GACATCCAGATGACCCAGTCTCCATCTTCCGTGTCTGCATCTGTAGGAGACAGAG
TCACCATCACTTGTCGGGCGAGTCAGGGTATTAGCAGCTGGTTAGCCTGGTATCA
GCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAA
AGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCA
CCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTACTATTGTCAACAGGCTAA
CAGTTTCCCTCCAAGCACCTTCGGCCAAGGGACA

XhoI

<mark>CTCGAG</mark>

ATT AAA CGA

Constant domain κ

ACTGTGGCTGCACCATCT

--

From this sequence it can be seen that the restriction enzyme recognition site lies at the end of the signal sequence and that the first codon of the variable chain kappa coding sequence starts with GAC. Everything after the ApaLI restriction enzyme recognition site is part of the insert and when designing the primer for the PCR with which we attach these sites to the insert it is very important to stay "in frame". That is, DNA is read and translated into an amino acid three nucleotides at a time, which is called the "frame". If one nucleotide is lacking the DNA will be read in a different frame. Therefore, the AA nucleotides have been added right after the ApaLI site , they cause the variable chain to be read "in frame". The XhoI site is located such that the first three nucleotides form one codon and the second three form another codon. Right after the XhoI site there are three more amino acids and thereafter the constant domain

of the light chain begins. The M13 Reverse priming site is located in the vector before the signal sequence and allows sequencing of the vector at the site of the light chain.

For those cells which have a λ light chain we mutated a Pces1 vector which has the lambda constant domain instead of the kappa constant domain. We started out with a Pces1 vector with the lambda constant domain in it instead of the kappa domain, but unfortunately it lacked a restriction enzyme recognition site at the end of the variable domain. As shown here:

```
            ----------------------><-----CDR3---
361         TGAGGATGAGGCTGATTATTATTGCTGGTCATTTGCAGGCTCCTATTATGTCTTCGGGAC
103           E  D  E  A  D  Y  Y  C  W  S  F  A  G  S  Y  Y  V  F  G  T


            ----------------------->------constant lamda IGLC1*02------
421         AGGGACCGACGTCACCGTCCTCGGTCAGCCCAAGGCCAACCCCACTGTCACTCTGTTCCC
123           G  T  D  V  T  V  L  G  Q  P  K  A  N  P  T  V  T  L  F  P
```

Therefore, we mutated the vector at the CTCGGT site to yield a CTCGAG, which is the XhoI site. For mutation technique, see below.

The ligation of the heavy chain is different. The SfiI site is in the signal sequence and quite large, followed by two other codons which belong to the signal sequence. Therefore, to clone the insert into the vector a lot of nucleotides need to be added to the insert using PCR.

--

Signal sequence

ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGC

SfiI

GGCCCAGCCGGCC ATG GCC

Variable domain of the heavy chain

CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGACACCCTG
TCCCTCACCTGCGCTGTCTCTGGTGACTCCCTCGGTAGTTACTACTGGACCTGGAT
CCGACAGCCCCCCGGGAAGGGACTGGAATGGGTGGGTTATATCTATTACAGTGG
GAGCACCAATTATAACCCTTCCCTCAAGAGTCAAGCCGTCATATCAATCGACACA
ACCAACAACCGGTTCTCCCTGAACCTCAGCTCTGTGACCGCTGGAGACACGGCCG

TGTATTATTGTGTGAGAGGAAACGGCGCGCTCCTATCCTGGGGCCAAGGAACCCT
GGT

BsteII

<mark>GGTCACC</mark>

GTCTCAAGC

Constant domain of the heavy chain
GCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCT
CTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTCCCCGAACCGGG
AACGGTTTATTTACA

--

For all of our cloning we used so called "midi"-preparations. As described under "Preparation of DNA in "mini"-quantities using the TENS protocol" it is possible to grow cultures of bacteria and then isolate plasmid DNA from these bacteria. In the aforementioned method we used only 5 ml of culture to prepare DNA which yields a "mini" quantitity of DNA. During ligation of an insert into a vector it is necessary to have a certain optimal molar ratio between these two reactants in the ligation mix. Therefore mini preparations are not suited for ligation reactions using the Pces1 vector. This is for a number of reasons. First, DNA quantities are very low in a mini preparation because little culture is used. Furthermore, the Pces1 vector is a "low-copy" plasmid, which means that DNA yield is lower because the plasmid does not replicate so fast in the cytoplasm of bacteria, which further decreases DNA yield. Because the DNA quantities are so low it is hardly possible to measure the DNA quantity which makes it more difficult to reach an optimal molar ratio of insert to vector.

Therefore we prepared "midi"-preparations. Midi preparations are DNA preparations where about 50 ml of culture is used to prepare DNA and it usually yields a total of 20-100 µg of DNA, compared to a maximum of 20 µg for the mini preparations.

We used the "Plasmid DNA Purification" kit from Machery-Nagel (Düren, Germany), to isolate the plasmid DNA from the bacteria in culture. This kit uses a procedure similar to the TENS-method, where bacteria are lysed, freeing the plasmid DNA. This kit also contains

columns to which the DNA binds and after various washing and elutions steps a pure, salt- and protein free DNA solution results.

Midi DNA Preparation

We used the following protocol for Midi preparations:

1. Prepare an overnight culture with the bacterium containing the plasmid of interest
2. Centrifuge for 15 minutes at 4°C at 6000 g
3. Resuspend the pellet in 8 ml buffer S1
4. Add 8 ml buffer S2 and mix gently by inverting tube 6-8 times
5. Add 8 ml pre-cooled (4°C) buffer S3 mix by inverting 6-8 times and incubate on ice for 5 minutes
6. Equilibrate a Nucleobond™ column with buffer N2, 2.5 ml
7. After passing the lysate from step 5 through a filter, load the lysate on the column and allow the column to empty by gravity flow
8. Wash the column with 12 ml buffer N3
9. Elute the plasmid DNA with 5 ml buffer N5
10. Add 3.5 ml room-temperature isopropanol to precipitate the eluted plasmid DNA and centrifuge at 15000g for 30 minutes at 4°C
11. Discard supernatant and add room-temperature 70% ethanol and again centrifuge
12. Remove ethanol and allow pellet to dry
13. Resuspend pellet in water of an appropriate volume

We used this protocol to prepare DNA for the Pces1 vector. The heavy and light chain inserts were cloned into TOPO again, after which cultures were prepared. From these cultures MIDI preparations were made using the protocol above. These MIDI preparations were then digested and ligated as described above, which resulted in the inserts being cloned into the Pces1 vector at appropriate locations.

DNA mutation

We used the quikChange™ Site-directed mutagenesis kit from Stratagene for DNA mutation of the Pces1 vector.

The principle is as follows. First primers are constructed which incorporate the mutation. Using these primers, a PCR is run with a DNA polymerase from the kit which causes the newly synthesised strand to have staggered nicks. The newly synthesised strand therefore is different than the template strand. This allows the template strand to be digested by Dpn I, an enzyme, leaving the newly synthesised strand with the mutations in them available for transformation into competent bacterial cells where double stranded DNA is again formed.

This is shown in the following picture.
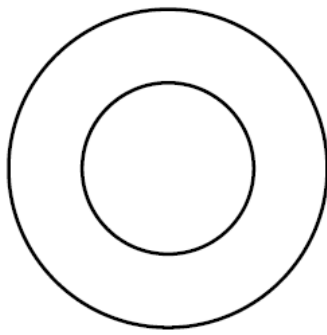
double stranded DNA
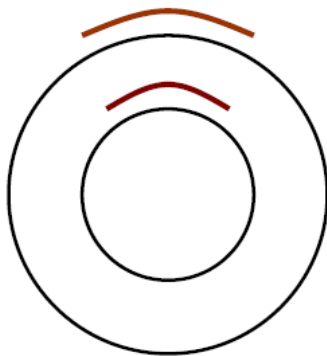
primer annealing
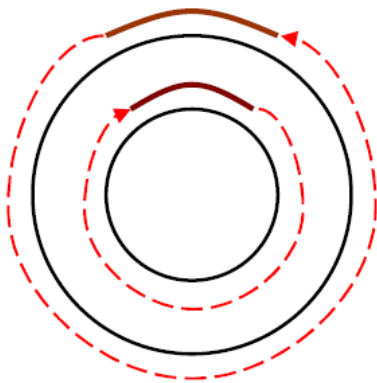
DNA replication
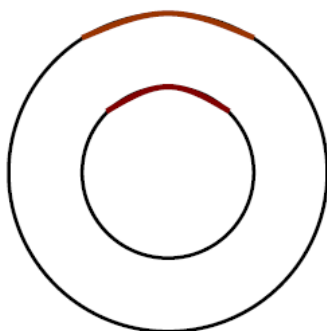
after digestion of original strand

Figure 27:

DNA mutation

We constructed two primers which anneal to opposite strands of the vector and which contain the desired mutation, CTCGGT to CTCGAG in our case. The primers were constructed so that they were between 25 and 45 bases long with a Tm above 78°C.

```
Mutprim5 CGTCACCGTCCTC GAG CAGCCCAAGGCC (84°C)
mutprim3 GGCCTTGGGCTG  CTC GAGGACGGTGACG (84°C)
```

Thereafter we ran a PCR using the provided *PfuTurbo* DNA polymerase using this protocol:

10x Reaction buffer	5
Pces1 Vector	2,5 (25 ng)
Mutprim5	1,5 (125 ng= 15 pmol)
Mutprim3	1,4 (125 ng= 14 pmol)
dNTP Mix	1
H2O	38,6
	--
	50

PfuTurbo DNA polymer	1

We used the following cycling parameters:

Step 1 Activation:	0.5 min 95 Grad
Step 2 Denaturation:	0.5 min 95 Grad
Step 3 Annealing:	1 min 55 Grad
Step 3 Extension	3.5 min 68 Grad
Step 4 Repeat	goto 2 18 times
Step 6 72 Grad 7 Minuten	
Step 7 Hold at 4°C forever	

We then did the DpnI digestion and transformed into E.Coli TG1 competent cells, picked colonies, grew cultures, isolated DNA and sent this DNA for sequencing to check if the mutation was successful.

3.5 Production of Fabs using the pCes1 vector

As described in chapter 2.6 "Phage display technology" and chapter 3.2 "Overview of methods" soluble antibody fragments were produced by phagemid containing bacteria in low glucose medium to which IPTG has been added. These antibody fragments are directed to the periplasm because of the signal sequence as described above. The following protocol was used for preparation of these periplasmic antibody fragments (Fabs).

1. A 50 ml culture of 2TY-AG is inoculated with the bacterium which contains the phagemid of interest and grown overnight at 30°C while shaking
2. 7.5 ml of this overnight culture is inoculated in 750 ml fresh 2TY containing 100 µg/ml ampicillin and 0.1% glucose and grown at 37°C while shaking at 180 rpm
3. At OD_{600} of 0.8 - 0.9 add 1mM IPTG and continue to grow at 30°C for 4 hours while shaking at 180 rpm
4. Centrifuge cells down in 500 ml Beckman containers using no more then 400 ml capacity at a time for 15 minutes at 4000 rpm
5. Discard supernatant and freeze the cells at -20 overnight

The freezing step has shown to give a higher protein yield.

6. Lysing of pellet: thawed pellet is lysed with TES (pH 8.0) and the following proteaseinhibitors from Sigma corporation:
   - PHSF (Phenyl Methyl Sulfyl Fluorid) 2 mM
   - Pepstatin 2 mM
   - Leupeptin 10 mM
   - Benzamidin 2 mM
   - Chymostatin 2.5 µg/ml

7. Dialyse overnight to remove EDTA from TES Solution against PBS pH 8.0

Thereafter Immobilised Metal Affinity Chromatography (IMAC) was used to isolate the Fabs from the solution of lysed pellet using the HIS-tag, which is present in the Fabs. The HIS-tag

binds to the affinity column because the column is coated with $NiSo_4$, which binds the HIS-tag.

8. First, the column was equilibrated with 50 ml PBS
9. Then the column was loaded by filtration across the column of 10 ml 100 mM $NiSo_4$
10. The column was washed with 30 ml PBS
11. The pellet lysate was then filtered across the column at 4 ml/min with a peristaltic pump
12. The column was washed with PBS
13. Then the column was washed with continuously increasing imidazol concentration (0-150 mM in 20 steps of 5 ml), this is the elution step.

The Fabs were eluted at an imidazol concentration of about 70 mM.

14. The Fab-fraction was dialysed against PBS
15. Measuring of protein concentration at 280 nM and control of the Fab-fraction in a Coomassiegel was performed

3.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

Once Fab Fragments had been produced we used SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), as described by Sambrook[26], to investigate these proteins and prove they were intact. The SDS-PAGE method allows separation of proteins on the basis of molecular weight using a gel in an electrical field. By using a reducing agent, which breaks disulfide bridges, it is possible to further analyse the subunits of a certain protein. In our case this was useful because the heavy and light chain of a Fab fragment are held together by a disulfide bridge.

The principle is as follows. First, the proteins which are to be investigated, Fab fragments in our case, are denatured through incubation with SDS. This causes denaturation through breakage of hydrogenbridges removing secondary and tertiary structures rendering the protein in its most simple, rod-like, polypeptide structure. The denatured polypeptides bind

SDS and thus become negatively charged allowing separation in an electrical field. The SDS molecule consists of a tail of 12 carbon atoms attached to a hydrophilic sulphate group, giving the molecule amphiphilic properties which causes it to act as a detergent.
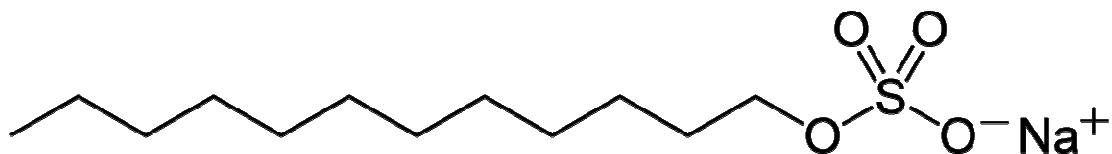
Figure 28:

Sodium dodecyl sulfate, $C_{12}H_{25}SO_4Na$

Approximately 1.4 gram of SDS is bound per gram of polypeptide. This causes the charges of the polypeptide to become negligible in comparison to the negative charge of the coating SDS. Effectively, because of denaturation and coating with SDS the proteins are rendered into uniformly negatively charged rod-like structures ideally suited for separation on a gel on the basis of size only using an electrical field. The polypeptides are separated in a polyacrylamide gel consisting of a mixture of bicrylamide and acrylamide in a ratio of 1:29 which yields an optimal separation based on molecular weight. The effective range of separation depends on the concentration of polycrylamide in the separating gel. SDS does not break disulfide bridges, to break disulfide bridges a reducing agent is required.

Practically, for our experiments, the SDS-PAGE of proteins was conducted as follows.
For descriptions of buffers used see "Kit contents, media and solutions" at the end of this chapter. First, we prepared a 12% polycrylamide gel. Then the gel was poured between two glass plates separated by about a millimetre, which hold the gel. The gel is left to polymerize for 30 minutes. After 30 minutes the top of the gel was washed with deionized water. Thereafter the stacking gel was loaded on top of the polyacrylamid gel, which serves as a holding gel for the proteins which are to be separated. Immediately after loading the stacking gel, before polymerization occurs, a comb was applied into the stacking gel to create the slots into which the proteins to be separated are loaded. The stacking gel was allowed to polymerize.

During this time our Fab Fragments were prepared for electrophoresis using two different procedures. We prepared a solution with Fab Fragments in a concentration of 100 µg/ml in

PBS and incubated with SDS gel-loading buffer for denaturation, whose principle is described above. We also prepared our samples under reducing conditions which means that in addition to incubation with SDS gel-loading buffer we also cooked the samples at 95° C followed by incubation with the β-mercaptoethanol reducing agent for 5 minutes.

After polymerization is complete the wells created by the comb were washed with deionized water. The wells were loaded with the samples containing the denatured and reduced proteins. Therafter the eletrophoresis was performed by applying an electrical field across the gel with a voltage of 180 V applied over a period of one hour.

After this the polyacrylamid gel containing the protein of interest was fixed and stained using a mixture of 0.25 g Coomassie Brilliant Blue in 90 ml Methanol:H_2O (1:1) and 10 ml of glacial acetic acid. The gel was incubated with this staining and fixing solution for 4 hours at room temperature. Thereafter the gel was immersed in the Methanol/H2O/acetic acid solution for a number of hours until an adequate destaining of the gel with appearance of proteinbands was attained. The gel obtained through this method was photographed.

Thereafter the gels were used for Western blotting as described below.

3.7 Western Blotting

Using the polyacrylamid gel obtained as described above we performed a Western blot to further prove the integrity of the Fab fragments we produced. Western Blotting is a technique in which the proteins which have been separated by SDS-PAGE are transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane to be incubated with antibodies that recognize certain polypeptide sequences (antigens). The last antibody in the antibody cascade is coupled to an enzyme which allows staining when incubated with an appropriate substrate.

The proteins in the polycrylamide gel are transferred to the membrane by placing the two media next to eachother in an electrical field sandwiched between two sheets of Whatman 3MM paper soaked in a transfer buffer which consists of Tris, glycine, SDS and methanol.

The transfer of the proteins from the gel takes about two hours and the successful completion of this procedure can be checked by staining the proteins on the membrane using Ponceau S, which does not interfere with further immunological probing because it is easily washed away in subsequent steps. The membrane was incubated with a working solution of Ponceau S, thereafter the membrane was washed with deionized water to make the bands visible. Thereafter the filter was washed with PBS to remove the Ponceau from the membrane after which further immunological probing can take place. In addition to the method of transfer above we also used the "semi-dry" method which is basically the same but differs in that only the Whatman papers are soaked with transfer buffer, the eletrophoresis using the semi-dry method took 30 minutes at 21 V.

We used a PVDF membrane and it was incubated with 10% milk powder at 4° C overnight, to block unspecific binding of antibody to the membrane. We then incubated with the primary and secondary antibody, whereby the second antibody is coupled to a peroxidase which is responsible for the staining of the nitrocellulose membrane in the presence of a substrate.

We used two pairs of primary and secondary antibodies. The first pair which consists of mouse anti-Penta-His IgG (Qiagen, Hilden, Germany) followed by goat anti-mouse IgG-HRP-conjugate (Biorad, Hercules, CA, USA) as secondary antibody, used in a concentration of 1:2000 and 1:3000 respectively. This first pair of antibodies reacts against the series of histamin peptides in the Fab fragment heavy chain and is therefore, in effect, an anti heavy-chain cascade. The second pair of antibodies we used consisted of rabbit anti-human-lambda IgG (Dako, Glostrup, Denmark) and goat anti-rabbit IgG-HRP-conjugate (Biorad, Hercules, CA, USA), used both in a concentration of 1:3000. This second pair of antibodies is, in effect, an anti light-chain cascade.

3.8 Enzyme-Linked ImmunoSorbent Assay (ELISA) for Fab testing against PR3

The principle of ELISA is as follows. First, a plastic plate with multiple wells, with one well for each probe or test to be performed, is coated with a certain target-antigen. The plate is made up of material to which proteins readily bind. After overnight coating with the antigen, the antigen is fixed to the plate and the coating solution is washed off. Thereafter, the plate is incubated with a serum or a solution containing an antibody (either a full antibody, a Fab or

even a Fab-expressing phage-particle). This is called the primary antibody. When the primary antibody specifically binds the target-antigen it will not be washed off in the subsequent wash-steps. Unbound antibody is washed off and the plate thereafter is coated with an enzyme-linked antibody against the primary antibody. After more washing the plate is incubated with the substrate for the enzyme and the intensity of coloring after a certain timeperiod of incubation is related to the amount of primary antibody which bound the target-antigen. This is therefore a measure for the strength of binding of the primary antibody to the target-antigen. Aspecific binding is prevented by "blocking" with a solution of milk powder.

We used NUNC Maxisorb™ 96-well plates.

We use this technique to test our Fabs for binding against PR3.

We used the following protocol for ELISA:

1. Coat plates for 2 hours with target-protein with a protein concentration of 2 µg/ml at room temperature
2. Wash 3 times with PBS
3. Incubate each well with 100 µl Fabs in 1% BSA (bovine serum albumin) in PBS
4. Incubate for 1 hour
5. Wash 3 times with PBS-Tween 0.05%
6. Wash 3 times with PBS
7. Add 100 µl of the primary antibody in 1% BSA
8. Incubate for 1 hour
9. Wash 3 times with PBS-Tween 0.1%
10. Wash 3 times with PBS
11. Add 100 µl of peroxidase coupled secondary antibody 1:2000
12. Incubate for 1 hour
13. Wash three times with PBS-Tween 0.1%, wash 3 times with PBS
14. Add 100 µl TMB solution per Well and incubate for 10 minutes
15. Add 50 µl Stopsolution per Well
16. Measure OD_{450}

As a primary antibody we used the Immunostep™ (Salamanca, Spain), Mouse monoclonal antibody to human lambda 0,1 mg/ml in diluted 1:500. As a secondary antibody we used DAKO™ (Glostrup, Denmark), polyclonal rabbit anti-mouse immunoglobulins. This secondary antibody was coupled to HRP: horse radish peroxidase. This secondary antibody was diluted 1:2000. The stop solution consisted of 1M H_2SO_4.

3.9 Kit contents, Media and solutions

Contents of the Zero Blunt TOPO PCR cloning kit

pCR-Blunt II-TOPO	10 ng/µl plasmid DNA in:
	50% glycerol
	50 mM Tris-HCL, pH 7.4 (at 25° C)
	1 mM EDTA
	2 mM DTT
	0.1% Triton X-100
	100 µg/ml BSA
	30 µM bromophenol blue
Salt Solution	1.2 M NaCl
	0.06 M $MgCl_2$

Contents of the Plasmid DNA purification kit by Machery-Nagel

Buffer S1	50 mM Tris-HCl
	10 mM EDTA
	100 µg/ml RNase A, pH 8.0
Buffer S2	200 mM NaOH, 1% SDS
Buffer S3	2.8 M KAc, pH 5.1
Buffer N2	100 mM Tris, 15% ethanol, 900 mM KCl, 0,15% Triton X-100, adjusted to pH 6.3 with H_3PO_4
Buffer N3	100 mM Tris, 15% ethanol, 1.15 M KCl, adjusted to pH 6.3 with H_3PO_4
Buffer N5	100 mM Tris, 15% ethanol, 1 M KCl, adjusted to pH 8.5 with H_3PO_4

Nucleobond™ columns

Solutions used for making competent cells

TB-solution (CaCl$_2$-solution)

10 mM PIPES (C$_8$H$_{18}$N$_2$O$_6$S$_2$) (3.021 g/l)

55 mM MnCl$_2$ (MnCl$_2$ 4 H$_2$O 10.885 g/l)

15 mM CaCl$_2$ (CaCl$_2$ 2H$_2$O 2.205 g/l)

250 mM KCl (18.637 g/l)

Mix and adjust pH to 6.7 with KOH

Sterilization by filtration across a 0.45 µm filter

SOC Medium

2% bactotryptone

0.5% Yeast extract

10 mM NaCl

2.5 mM MgCl$_2$

10 mM MgSO$_4$

20 mM Glucose

Solutions used in the TENS-method

TENS-Buffer

Solution with:

1% Tris 1M

1% 10 M NaOH

5% SDS 10%

In water

NaAc 3M pH 5.2

Dissolve 408.1 g of NaAc·3H$_2$O in 800 ml H$_2$O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 liter, autoclave

Solutions used in SDS-PAGE

12% Polyacrylamide gel (20 ml)

6.6	ml	H$_2$O
8 .0	ml	30% Acrylamide mix
5.0	ml	1.5 M Tris (pH 8.8)
0.2	ml	10% SDS
0.2	ml	10% Ammonium persulfate
0.008	ml	TEMED

Acrylamide mix	29% acrylamide and 1% methylenebisacrylamide in deionized H_2O
TEMED	N,N,N´,N´-tetramethylethylenediamine (polymerization agent)
5% Stacking Gel (10 ml)	H_2O 6.8 ml 30% Acrylamide mix 1.7 ml 1.0 M Tris (pH 6.8) 1.25 ml 10% SDS 0.1 ml 10% Ammonium persulfate 0.1 ml TEMED 0.01 ml
1x SDS gel-loading Buffer	50 mM Tris·Cl (pH 6.8) 100 mM dithiothreitol 2% SDS 0.1% bromophenol blue 10% Glycerol

Media used for Western blotting

Transfer Buffer	39 mM glycine 48 mM Tris base 0.037% SDS 20% methanol To prepare 1 liter of transfer buffer (pH 8.3), mix 2.9 g of glycine, 5.8 g of Tris base, 0.37 g of SDS and 200 ml of Methanol
Stock solution of Ponceau S	Ponceau S 2 g Trichloracetic acid 30 g Sulfosalicylic acid 30 g H_2O to 100 ml

Working solution of Ponceau S	1:10 dilution of Stock solution using deionized water

Media and solutions

LB-medium (with Kanamycin)	1% Bactotryptone
	0.5% Yeast extract
	1.0% NaCl
	pH 7.0
	autoclave
	(Add 50 µg/ml Kanamycin)
LB-Agar plates with Kanamycin	LB-medium
	Add agar 15 g/L
	Autoclave
	Add 50 µg/ml Kanamycin
	Pour into plates with diameter of 10 cm
	Store at 4°C.
Tris-acetate 50x (TAE)	242 g Tris base
	57.1 ml glacial acetic acid (CH_3COOH)
	100 ml 0.5 M EDTA (pH 8.0)
TE-Buffer pH 7.4	10 mM Tris·Cl (pH 7.4)
	1 mM EDTA (pH 80)
Tris 1M	121.1 g Tris Base in 800 ml H_2O
	Adjust pH by adding HCl
	pH 7.4 70 ml
	pH 7.5 60 ml
	pH 8.0 42 ml
SDS 10%	100 g SDS in 900 ml H_2O. Heat to 68°C to aid in dissolution. Adjust pH to 7.0 with

a few drops of 10M NaOH solution, adjust volume to 1 liter. Autoclave.

PBS
8.75 g NaCl / liter water (750 mmol / liter)
1.42 g Na_2HPO_4 / liter water (40 mmol/liter)
0.22 g KH_2PO_4 / liter water (7.8 mmol /liter)
pH should be approximately 7.4-7.6

PEG/NaCl
20% Polyethylene glycol 6000 (PEG)
2.5 M NaCl
Autoclave

2xTY broth
16 g bacto-tryptone / liter water
10 g Yeast extract / liter water
5 g NaCl / liter water

2xTY-AG
2xTY broth, containing 100 µg/ml ampicillin and 2 % glucose

2xTY-AG plates
2xTY broth, containing 2% glucose, 100 µg/ml ampicillin and 15 g agar

4. Results

4.1 Cloning of DNA coding for the variable heavy and light chain domains of antibodies into the Pces1 vector

Table 6: Designations of two B-cells derived from two WG patients and their light-chain subclass

B-cell	Light chain
St140	K
Scu68	Λ

Each light and heavy chain was cloned into the TOPO vector, the vector was transformed into DH5-α bacteria and plated out onto LB/Kanamycin plates. From each product ten colonies were picked and sequenced. This was done to check for monoclonality of the product, since it is possible that despite careful handling more than one cell is lasered out of the tissue section. If the product is not monoclonal, multiple insert will be found, and consecutively not all of the 10 sequences will be identical. The cells that were used for further investigations were monoclonal.

The sequences were analyzed using the IMGT immunoglobulin database. The results of these analyses yielded the families to which the particular heavy and light chain belonged to and for the light chain it allowed determination of κ- or λ-type.

The results are shown in appendix I. The most important result which is to be noted is that most of the heavy-chain coding DNA sequences had to be extended to be used directly for cloning into the Pces1 vector.

An example is shown below, it is the sequence of st140.

It can be seen that the sequence bears the greatest resemblance to the 4-59 family of DNA-segment coding for the variable heavy chain of the antibody. The first 18 codons bridging the insertion site of the vector's are missing. For our purposes this is not so important since the antibody specificity is determined by the complementary determining regions which is located 3' of the variable domain coding DNA.

St140 VH

```
                            <---------------------------------------- FR1 - IMGT
                            1             5              10             15
st140vh                     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L10088 IGHV4-59*01          cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
M29812 IGHV4-59*02          cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
X56360 IGHV4-59*07          cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
M95114 IGHV4-59*03          cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
X87091 IGHV4-59*08          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


                            ---------------------------------------->  _____
                            20             25             30
st140vh                     ... ... ..c ctg tcc ctc acc tgc gct gtc tct ggt gac tcc ctc
L10088 IGHV4-59*01          tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
M29812 IGHV4-59*02          tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- g--
X56360 IGHV4-59*07          tcg gac ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
M95114 IGHV4-59*03          tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
X87091 IGHV4-59*08          ... ... ... ... --- --- --- --- a-- --- --- --- -g- --- a--


                            __   CDR1 - IMGT  _____  <------------------------
                                 35             40             45
st140vh                     ggt agt tac tac ... ... ... ... tgt acc tgg atc cga cag ccc
L10088 IGHV4-59*01          a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
M29812 IGHV4-59*02          a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
X56360 IGHV4-59*07          a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
M95114 IGHV4-59*03          a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
X87091 IGHV4-59*08          a-n --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
```

As described in "material and methods" we need the complete DNA segment which codes for the variable domain of the heavy and light chain to clone into the Pces1 vector to be able to produce fabs. For the light chain, the restriction enzyme recognition site is situated at the beginning of the DNA coding for the variable chain and for the heavy chain the recognition site is situated in the signal sequence some codons 5' of the DNA coding for the variable chain. Both the missing codons and the restriction enzyme recognition site needed to be added to the variable domain coding DNA segments.

For each DNA segment where codons were missing we used the germline sequence to fill it up. In the example above, the St140Vh is most similar to the V4-59 family. Thus the particular variable heavy segment bears the highest similarity to the V4-59 family in its non-recombinant form, and additional mutations are acquired by affinity maturation. This germline sequence is shown in the IMGT analysis above. The addition of germline homologue gene segments to extend the framework region up to the insertion sequence that fit to the vector ligation should not change the affinity of the Fab or antibody produced since the complementarity-determining regions are not affected.

In the example above the missing 18 codons were added. 5' from these codons, a part of the signal sequence containing the SfiI restriction enzyme recognition site also was added, representing another 19 nucleotides. This is illustrated here.

Signal sequence

ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGC

SfiI

==GGCCCAGCCGGCC== ATG GCC

Variable domain of the heavy chain

CAGGTGCAGCTGCAGG etc. etc. etc.

For the heavy chain a total of about 20-24 codons were added, depending on how many codons were missing. These codons were added using PCR with "overhanging" primers, as was described in "materials and methods". Twenty-four codons are 72 nucleotides to be added at once. The primer itself will exceed this length because it also has to anneal to the DNA. This represents a problem since the maximum length of primers which can be easily produced in commercial laboratories is 50 basepairs. Consecutively, it is not possible to add all of the missing nucleotides to our DNA inserts at once. We therefore chose to do multiple rounds of PCR, adding seven codons in each round. This requires different primers for each round. The first primer anneals to the DNA present in the insert, the second primer anneals to the codons which have been added to the insert in the first round of PCR by the first primer and adds more codons to which the primer in the third round attaches etc. The primers thus have an overlap, otherwise annealing could not take place.

This is illustrated in the following picture.
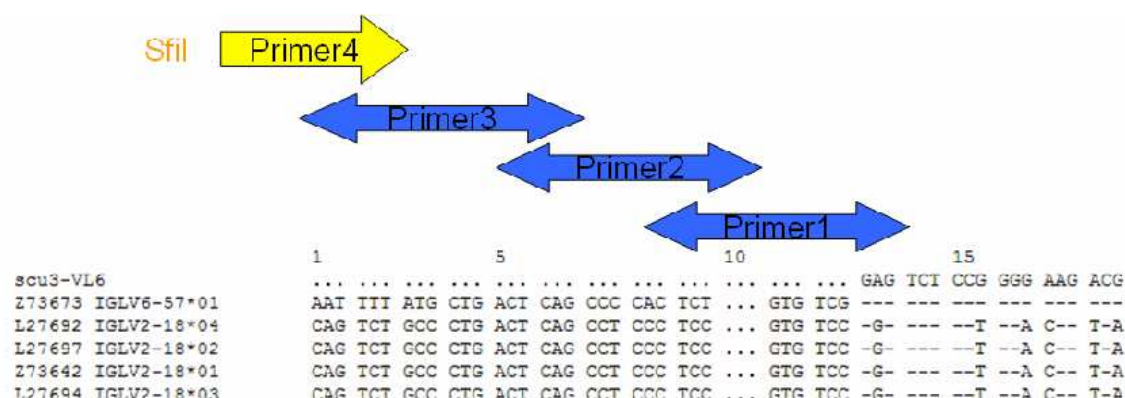
Figure 30:

Addition of germline DNA and restriction enzyme recognition sites, with overlapping primers

For each of the DNA segments listed in Appendix I primers were devised and they are shown here. The 3' primer is never divided into multiple primers because at the end of the DNA sequences there were no missing codons and therefore this primer was used in each round of PCR using the 5' primers. The "theoretical primer" is the primer sequence if we were able to add all the required nucleotides in one step. Parentheses () are used to indicate where the division is located between nucleotides in the primer which anneal and which nucleotides are added. The nucleotides to the left of the parenthesis are added in that particular round. 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 purposely chose the restriction enzyme recognition sites in such a way that as much of the original DNA sequence was spared so as not to alter the binding properties of the immunoglobulin. The results of these considerations are shown below.

St140 vh
```
Theoretical primer:
ggg ggg GCG GCC CAG CCG GCC ATG GCC (end of signal sequence) CAG GTG CAG
CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAC AC()C CTG TCC CTC ACC
TGC GCT GTC (74 Grad)
```

Divided into:

```
5'-first
CTG GTG AAG CCT TCG GAC AC ()C CTG TCC CTC ACC TGC GCT GTC (72°C)


5'-second
CTG CAG GAG TCG GGC CCA GGA ()CTG GTG AAG CCT TCG GAC AC C C (72°C)


5'-third
CCG GCC ATG GCC CAG GTG CAG ()CTG CAG GAG TCG GGC CCA GG (70°C)


5'-fourth
ggg ggg GCG GCC CAG()CCG GCC ATG GCC CAG GTG CA (70°C)
```

3'-primer

```
GGG GGG ()GGTGACCACCAGGGTTCCTTGGCCCCAGG
```

St140vk

```
5'-primer
GTG CAC AA GAC ATC CAG ATG ACC ()CAG TCT CCA TCT TCC GTG TCT GC (72°C)


3'-primer
CTC GAG T () GTCCCTTGGCCGAAGGTGCTTG (72°C)
```

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)
```

Scu68vl

```
5`
GGG GGG GTGCAC () CCT TCT GAG CTG ACT CAG GAC CC (72°C)


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

As a rather novel and so far not yet used method we did not perform the PCRs separately, but we mixed the primers together in one PCR. The theory behind this is that as soon as the first primer has bound to the target DNA sequence and some cycles have been performed the nucleotides which are supposed to be added by the first primer are actually added, creating the target DNA molecule to which the second primer can attach which leads to addition of more nucleotides to which the third primer can bind. Of course, the chance of unspecific primer binding and primers binding to eachother increases because in our experiments a maximum of 5 different primers were present in the PCR mix (four 5' primer and the 3' primer).

To investigate the results of each of these mixed-primer PCRs the PCR products were cloned into the TOPO vector and transformed into bacteria for sequencing of selected clones. The results of this mixing of primers was very different for each PCR which was tried. Two out of three PCRs were successful in that all the primers had bound appropriately leading to an intact PCR product. In one of three PCR reactions the fourth primer had not bound. For scu68VH none of ten PCR products contained the right sequence, which forced us to run the PCRs in consecutive rounds.

As a result of these efforts we had the complete DNA sequence of the light and heavy chain coding DNA-segments. This is shown below:

<u>St140 VH</u>

```
                           <---------------------------------------- FR1 - IMGT
                           1               5                 10                  15
St140VH                    cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
X56360 IGHV4-59*07         --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
L10088 IGHV4-59*01         --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M29812 IGHV4-59*02         --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M95114 IGHV4-59*03         --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M95117 IGHV4-59*04         --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
```

```
                      ------------------------------------------> _____
                      20                  25                  30
St140VH               tcg gac acc ctg tcc ctc acc tgc gct gtc tct ggt gac tcc ctc
X56360  IGHV4-59*07   --- --- --- --- --- --- --- --- a-- --- --- --- -g- --- a--
L10088  IGHV4-59*01   --- --g --- --- --- --- --- --- a-- --- --- --- -g- --- a--
M29812  IGHV4-59*02   --- --g --- --- --- --- --- --- a-- --- --- --- -g- --- g--
M95114  IGHV4-59*03   --- --g --- --- --- --- --- --- a-- --- --- --- -g- --- a--
M95117  IGHV4-59*04   --- --g --- --- --- --- --- --- a-- --- --- --- -g- --- a--


                      __  CDR1 - IMGT _____ <------------------------
                      35                  40                  45
St140VH               ggt agt tac tac ... ... ... ... tgg acc tgg atc cga cag ccc
X56360  IGHV4-59*07   a-- --- --- --- ... ... ... ... --- -g- --- --- --g --- ---
L10088  IGHV4-59*01   a-- --- --- --- ... ... ... ... --- -g- --- --- --g --- ---
M29812  IGHV4-59*02   a-- --- --- --- ... ... ... ... --- -g- --- --- --g --- ---
M95114  IGHV4-59*03   a-- --- --- --- ... ... ... ... --- -g- --- --- --g --- ---
M95117  IGHV4-59*04   a-- --- --- --- ... ... ... ... --- -g- --- --- --g --- ---


                      FR2 - IMGT  ------------------------> _____  CDR2
                      50                  55                  60
St140VH               ccc ggg aag gga ctg gaa tgg gtg ggt tat atc tat tac agt ggg
X56360  IGHV4-59*07   --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
L10088  IGHV4-59*01   --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
M29812  IGHV4-59*02   --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
M95114  IGHV4-59*03   --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
M95117  IGHV4-59*04   --a --- --- --- --- --g --- a-t --g --- --- --- --t --- ---


                      - IMGT  _____ <-------------------------------------
                      65                  70                  75
St140VH               agc acc ... ... ... aat tat aac cct tcc ctc aag ... agt caa
X56360  IGHV4-59*07   --- --- ... ... ... --c --c --- --c --- --- --- ... --- -g-
L10088  IGHV4-59*01   --- --- ... ... ... --c --c --- --c --- --- --- ... --- -g-
M29812  IGHV4-59*02   --- --- ... ... ... --c --c --- --c --- --- --- ... --- -g-
M95114  IGHV4-59*03   --- --- ... ... ... --c --c --- --c --- --- --- ... --- -g-
M95117  IGHV4-59*04   --- --- ... ... ... t-c --c --- --g --- --- --- ... --- -g-


                      ----------------------------- FR3 - IMGT  ---------------
                      80                  85                  90
St140VH               gcc gtc ata tca atc gac aca acc aac aac cgg ttc tcc ctg aac
X56360  IGHV4-59*07   -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
L10088  IGHV4-59*01   -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
M29812  IGHV4-59*02   -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
M95114  IGHV4-59*03   -t- ac- --- --- g-a --- --g t-- --g --- -aa --- --- --- --g
M95117  IGHV4-59*04   -t- ac- --g --- g-a --- --g t-- --g --- -a- --- --- --- --g


                      ----------------------------------------------------> ____
                      95                  100                 104
St140VH               ctc agc tct gtg acc gct gga gac acg gcc gtg tat tat tgt gtg
X56360  IGHV4-59*07   --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
L10088  IGHV4-59*01   --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
M29812  IGHV4-59*02   --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
```

81

```
M95114 IGHV4-59*03          --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
M95117 IGHV4-59*04          --g --- --- --- --- --c -c- --- --- --t --- --- --c --- -c-


                            _____   CDR3 -  IMGT _____


St140VH                     aga gga aac ggc gcg ctc cta tcc tgg ggc caa gga acc ctg gtg
X56360 IGHV4-59*07          ---
L10088 IGHV4-59*01          --- -a
M29812 IGHV4-59*02          --- -a
M95114 IGHV4-59*03
M95117 IGHV4-59*04




St140VH                     gtc acc
X56360 IGHV4-59*07
L10088 IGHV4-59*01
M29812 IGHV4-59*02
M95114 IGHV4-59*03
M95117 IGHV4-59*04
```

St140vκ

```
                            <------------------------------------- FR1 - IMGT
                            1            5               10               15
st140Vk                     gac atc cag atg acc cag tct cca tct tcc gtg tct gca tct gta
V01577 IGKV1-12*01          --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02          --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1D-12*02         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01         --- --- --- --- --- --- --- --- --- --t --- --- --- --- ---
K01323 IGKV1D-16*01         --- --- --- --- --- --- --- --- --c --a c-- --- --- --- ---


                            ----------------------------------------> _____
                                         20              25               30
st140Vk                     gga gac aga gtc acc atc act tgt cgg gcg agt cag ggt att agc
V01577 IGKV1-12*01          --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02          --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1D-12*02         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
K01323 IGKV1D-16*01         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---


                            __  CDR1 - IMGT _____ <-------------------------
                                         35              40               45
st140Vk                     agc tgg ... ... ... ... ... ... tta gcc tgg tat cag cag aaa
V01577 IGKV1-12*01          --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
V01576 IGKV1-12*02          --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
V01576 IGKV1D-12*02         --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
X17263 IGKV1D-12*01         --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
K01323 IGKV1D-16*01         --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---


                            FR2 - IMGT ------------------------> _____ CDR2
                                         50              55               60
```

```
st140Vk              cca ggg aaa gcc cct aag ctc ctg atc tat gct gca tcc ... ...
V01577 IGKV1-12*01   --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
V01576 IGKV1-12*02   --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
V01576 IGKV1D-12*02  --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
X17263 IGKV1D-12*01  --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
K01323 IGKV1D-16*01  --- -a- --- --- --- --- tc- --- --- --- --- --- --- ... ...


                       - IMGT   _____  <----------------------------------------
                                 65                  70                  75
st140Vk              ... ... ... ... ... agt ttg caa agt ggg gtc cca ... tca agg
V01577 IGKV1-12*01   ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
V01576 IGKV1-12*02   ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
V01576 IGKV1D-12*02  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
X17263 IGKV1D-12*01  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
K01323 IGKV1D-16*01  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---


                     ----------------------------- FR3 - IMGT  --------------
                                 80                  85                  90
st140Vk              ttc agc ggc agt gga ... ... tct ggg aca gat ttc act ctc acc
V01577 IGKV1-12*01   --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02   --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
V01576 IGKV1D-12*02  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01  --- --- --- --- --- ... ... --- --- --- --- --- --- --- --t
K01323 IGKV1D-16*01  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---


                     ------------------------------------------------------> ____
                                 95                 100                 104
st140Vk              atc agc agc ctg cag cct gaa gat ttt gca act tac tat tgt caa
V01577 IGKV1-12*01   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02   --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1D-12*02  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
K01323 IGKV1D-16*01  --- --- --- --- --- --- --- --- --- --- --- --t --c --c ---


                     _____  CDR3 - IMGT  _____
st140Vk              cag gct aac agt ttc cct cca agc acc ttc ggc caa ggg aca ctc
V01577 IGKV1-12*01   --- --- --- --- --- --- --
V01576 IGKV1-12*02   --- --- --- --- --- --- t-
V01576 IGKV1D-12*02  --- --- --- --- --- --- t-
X17263 IGKV1D-12*01  --- --- --- --- --- --- --
K01323 IGKV1D-16*01  --- ta- --t --- -a- --- --



st140Vk              gag
V01577 IGKV1-12*01
V01576 IGKV1-12*02
V01576 IGKV1D-12*02
X17263 IGKV1D-12*01
K01323 IGKV1D-16*01
```

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   --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
X92230 IGHV4-61*03   --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
AB019437 IGHV4-61*08 --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
X92250 IGHV4-61*04   --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M29812 IGHV4-59*02   --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---


                ------------------------------------------> _____
                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- --- ---
X92230 IGHV4-61*03   --- --- --- --- --- --- --- --- a-- --- --- --t -g- --- ---
AB019437 IGHV4-61*08 --- --- --- --- --- --- --- --- a-- --- --- --t -g- --- ---
X92250 IGHV4-61*04   --- --- --- --- --- --- --- --- a-- --- --- --t -g- --- ---
M29812 IGHV4-59*02   --- --- --- --- --- --- --- --- 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--
X92230 IGHV4-61*03   agc ag- -g- -g- --c --c ... ... --- -g- --- --c --g --- c--
AB019437 IGHV4-61*08 agc ag- -g- gg- --c --c ... ... --- -g- --- --c --g --- c--
X92250 IGHV4-61*04   agc ag- -g- -g- --c --c ... ... --- -g- --- --c --g --- c--
M29812 IGHV4-59*02   agt ag- t-c tac ... ... ... ... --- -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 ---
X92230 IGHV4-61*03   --- --- --- --- --- --- --- --- --g t-- a-- --- --- --t ---
AB019437 IGHV4-61*08 --- --- --- --- --- --- --- --- --g t-- a-- --- --- --t ---
X92250 IGHV4-61*04   --- --- --- --- --- --- --- --- --a t-- a-- --- --- --t ---
M29812 IGHV4-59*02   --- --- --- --- --- --- --- --- --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-- ... --- ---
X92230 IGHV4-61*03   -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
AB019437 IGHV4-61*08 -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
X92250 IGHV4-61*04   -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
M29812 IGHV4-59*02   -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
X92230 IGHV4-61*03   --- --- a-a --a g-a --- --- --- --g --c --c --- --c --- --g
AB019437 IGHV4-61*08 --- --- a-a --a g-a --- --- --- --g --c --- --- --c --- --g
```

```
X92250 IGHV4-61*04          --- --- a-a --a g-a --- --- --- --g --c --- --- --c --- --g
M29812 IGHV4-59*02          --- --- 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-
X92230 IGHV4-61*03          --- --c --- --- --- g-- --- --- --- --- g-g --- -ac --- -c-
AB019437 IGHV4-61*08        --- --c --- --- --- g-- --- --- --- --- g-g --- -ac --- -c-
X92250 IGHV4-61*04          --- --c --- --- --. ..c --t --- --- --- g-g --- -ac --
M29812 IGHV4-59*02          --- --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          --- --
X92230 IGHV4-61*03          --- --
AB019437 IGHV4-61*08        --- --
X92250 IGHV4-61*04
M29812 IGHV4-59*02          --- --


                            ___

scu68VH                     ccc tgg ggc caa gga ccc ctg gtc aca ggg gtc acc gtc tca agc
M29811 IGHV4-61*01
X92230 IGHV4-61*03
AB019437 IGHV4-61*08
X92250 IGHV4-61*04
M29812 IGHV4-59*02
```

Scu68Vl

```
                            <------------------------------------------ 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-- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
Z73645 IGLV3-32*01(ORF)     t-c --- -g- -ca --- --- -tg --- --a ... --- --- --- --- ---
X57826 IGLV3-1*01           t-c -a- --- --- --- --- cca --c t-a ... --- --c --- t-- cca
X97473 IGLV3-9*01           t-c -a- --- --- --- --- cca -tc t-a ... --- --a --- --- c--
M94116 IGLV1-40*01          -ag --- -t- --- --g --- ccg --c t-a ... --- --- -g- --- cca


                            ------------------------------------------> _____
                                        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
Z73645 IGLV3-32*01(ORF)     --a --a -tg -c- -gg --- --c --- --- --- --- --- a-g g-- g-c
X57826 IGLV3-1*01           --a --- --- -c- -gc --- --c --- tct --- --t -aa t-g ggg gat
X97473 IGLV3-9*01           --a --- --g -c- -gg --t --c --t gg- --- a-- -a- a-t gg- --t
M94116 IGLV1-40*01          --- --- -gg --- --c --- t-c --- act --g ag- --- tc- --c -tc


                            __ CDR1 - IMGT _____ <-------------------------
                                        35              40              45
scu68Vl                     tat tat ... ... ... ... ... ... gca ggc tgg tat aag cag aag
```

85

```
X56178 IGLV3-19*01          --- --- ... ... ... ... ... --- a-- --- --c c-- --- ---
Z73645 IGLV3-32*01(ORF)     -c- --- ... ... ... ... ... -a- ca- --- --c c-- --- ---
X57826 IGLV3-1*01           a-a --- ... ... ... ... ... --t t-- --- --- c-- --- ---
X97473 IGLV3-9*01           a-a a-- ... ... ... ... ... -tg ca- --- --c c-- --- ---
M94116 IGLV1-40*01          ggg gca ggt tat gat ... ... ... -t- ca- --- --c c-- --- ctt


                            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- --- --- ... ...
Z73645 IGLV3-32*01(ORF)     --- --c --- --- --c gtg --g --- --c --- -a- -gc -gt ... ...
X57826 IGLV3-1*01           --- --c --- t-- --- gtg --g --- --c --- caa g-t -g- ... ...
X97473 IGLV3-9*01           --- --c --- --- --- gtg --g --- --c --- agg g-t -g- ... ...
M94116 IGLV1-40*01          --- --- aca --- --c a-- --c c-- --c --- -g- --c -g- ... ...


                             - IMGT  _____  <------------------------------------
                                65            70            75
scu68Vl                     ... ... ... ... ... agg cgg ccc tca ggg atc cca ... gac cga
X56178 IGLV3-19*01          ... ... ... ... ... -ac --- --- --- --- --- --- ... --- ---
Z73645 IGLV3-32*01(ORF)     ... ... ... ... ... gac --- --- --- a-- --- --t ... --g ---
X57826 IGLV3-1*01           ... ... ... ... ... -a- --- --- --- --- --- --t ... --g ---
X97473 IGLV3-9*01           ... ... ... ... ... -ac --- --- --t --- --- --t ... --g ---
M94116 IGLV1-40*01          ... ... ... ... ... -at --- --- --- --- g-- --t ... --- ---


                            ------------------------------  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 --- --- ---
Z73645 IGLV3-32*01(ORF)     --- --- --c --- --a ... ... --- --c a-- --- a-- a-- c-- ---
X57826 IGLV3-1*01           --- --- --c --- --- ... ... --t --g a-- --- --- a-t c-- ---
X97473 IGLV3-9*01           --- --- --c --- --- ... ... --g --g a-- --g --- a-- c-- ---
M94116 IGLV1-40*01          --- --- --c --- --g ... ... --t --c ac- t-- --- --- c-- g--


                            ------------------------------------------------------->  ____
                                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
Z73645 IGLV3-32*01(ORF)     --- --- --- --c --- --t --g --- --- --- --t --- -a- -a- c-g
X57826 IGLV3-1*01           --- -gc --- a-c --- --t atg --- --- --- --c --- -a- --- c-g
X97473 IGLV3-9*01           --- -gc a-a --c --a --c -gg --- --- --- --c --- -a- --- c-g
M94116 IGLV1-40*01          --- --- --- ctc --- --t --g --- --- --- --t --- -a- --c c-g


                            _____  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
Z73645 IGLV3-32*01(ORF)     -tg ata --- aa- ca- -ct -c
X57826 IGLV3-1*01           g-g t-g --- a-- agc act gca
X97473 IGLV3-9*01           gtg t-g --- a-- agc act gca
M94116 IGLV1-40*01          --- tat --- a-- agc ctg -g- gg- tc
```

```
scu68Vl                         acc aag ctg acc gtc ctc gag
X56178 IGLV3-19*01
Z73645 IGLV3-32*01(ORF)
X57826 IGLV3-1*01
X97473 IGLV3-9*01
M94116 IGLV1-40*01
```

For the ELISA against proteinase-3 we developed a positive control based on an article by J. Davis[3]. The immunoglobulin sequence coding for the variable heavy and light chain domains which is shown in this article is specific for proteinase-3. It was, however, not complete because the first 8 codons were missing. To produce a fab based on this sequence Doctor Csernok (Bad Bramstedt, Germany), kindly sent us a hybridoma which produces antibodies and which uses the sequence given in the aforementioned article. 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 coding for the heavy and light variable domains. This positive control is called "Pr3pos", it has a lambda light-chain and the primers are shown here:

Pr3pos heavy chain primers:
`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'-primer`
GGG GGG GAGGAGACGGTGACCAGGGTTC (74°C)

Pr3pos light chain primers

`5'-light chain`
GGG GGG G GTG CA () CAG TCT GCC CTG ACT CAG CCT G (72°C)

3'-light chain

GGG GGG CTCGAGGACGGTCAGCTTGGTCCCTCCG

The resulting sequence is shown below:

Sequence of the proteinase-3 positive control.

This sequence is based on Davis.[3]

Pr3posVH

```
                      <------------------------------------- FR1 - IMGT
                      1             5             10            15
Pr3posvh              cag gtg cag ctg gtg gag tct ggg gga ... ggc gtg gtc cag cct
M99663 IGHV3-30*03    --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
X92214 IGHV3-30*18    --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
L06616 IGHV3-30*19    --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M77334 IGHV3-33*05    --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
M77339 IGHV3-30*13    --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---


                      ------------------------------------------> _____
                      20            25            30
Pr3posvh              ggg agg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc
M99663 IGHV3-30*03    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X92214 IGHV3-30*18    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L06616 IGHV3-30*19    --- --- --- --- --- --- --- --- --- --g --- --- --- --- ---
M77334 IGHV3-33*05    --- --- --- --- --- --- --- --- --- --g --- --- --- --- ---
M77339 IGHV3-30*13    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---


                      __   CDR1 - IMGT  _____  <-------------------------
                           35            40            45
Pr3posvh              agt agc tat ggc ... ... ... ... atg cac tgg gtc cgc cag gct
M99663 IGHV3-30*03    --- --- --- --- ... ... ... ... --- --- --- --- --- --- ---
X92214 IGHV3-30*18    --- --- --- --- ... ... ... ... --- --- --- --- --- --- ---
L06616 IGHV3-30*19    --- --- --- --- ... ... ... ... --- --- --- --- --- --- ---
M77334 IGHV3-33*05    --- --- --- --- ... ... ... ... --- --- --- --- --- --- ---
M77339 IGHV3-30*13    --- --- --- --- ... ... ... ... --- --- --- --- --- --- ---


                      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    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X92214 IGHV3-30*18    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L06616 IGHV3-30*19    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
M77334 IGHV3-33*05    --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
M77339 IGHV3-30*13    --- --- --- --- --a --- --- --- --- --- --- --- --- --- ---


                      - IMGT  _____  <-------------------------------------
                              65            70            75
Pr3posvh              agt aat aaa ... ... tac tat gca gac tcc gtg aag ... ggc cga
```

```
M99663 IGHV3-30*03     --- --- --- ... ... --- --- --- --- --- --- --- ... --- ---
X92214 IGHV3-30*18     --- --- --- ... ... --- --- --- --- --- --- --- ... --- ---
L06616 IGHV3-30*19     --- --- --- ... ... --- --c --- --- --- --- --- ... --- ---
M77334 IGHV3-33*05     --- --- --- ... ... --- --- --- --- --- --- --- ... --- ---
M77339 IGHV3-30*13     --- --- --- ... ... --- --c --- --- --- --- --- ... --- ---


                       ----------------------------- 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     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X92214 IGHV3-30*18     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L06616 IGHV3-30*19     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
M77334 IGHV3-33*05     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
M77339 IGHV3-30*13     --- --- --- --- --- --- --- --- --- --- -g- --- --- --- ---


                       ------------------------------------------------------> ____
                               95             100             104
Pr3posvh               atg aac agc ctg aga gct gag gac acg gct gtg tat tac tgt gcg
M99663 IGHV3-30*03     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X92214 IGHV3-30*18     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L06616 IGHV3-30*19     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
M77334 IGHV3-33*05     --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
M77339 IGHV3-30*13     --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---


                       _____ CDR3 - IMGT _____
Pr3posvh               aag agc caa atg tcg tat tac gat ttt tgg agt ggt tat tac cgg
M99663 IGHV3-30*03     -ga ga
X92214 IGHV3-30*18     --a ga
L06616 IGHV3-30*19     -ga ga
M77334 IGHV3-33*05     -ga ga
M77339 IGHV3-30*13     -ga ga


                       _____
Pr3posvh               gac cag tac tac ttt gac tac tgg ggc cag gga acc ctg gtc acc
M99663 IGHV3-30*03
X92214 IGHV3-30*18
L06616 IGHV3-30*19
M77334 IGHV3-33*05
M77339 IGHV3-30*13
```

Pr3posVl

```
                       <---------------------------------------- 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     --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
L27822 IGLV2-14*02     --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
Z73665 IGLV2-23*02     --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
Y12412 IGLV2-14*03     ... ... ... ... ... ... ... ..- --- ... --- --- --- --- ---
D86994 IGLV2-23*03     --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
```

```
                         ------------------------------------------>  _____
                               20              25              30
Pr3posvl            gga cag tcg atc acc atc tcc tgc act gga acc agc agt gac gtt
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L27822 IGLV2-14*02  --- --- --- --- --- --- --- --- --- --- --- --- --- --t ---
Z73665 IGLV2-23*02  --- --- --- --- --- --- --- --- --- --- --- --- --- --t ---
Y12412 IGLV2-14*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
D86994 IGLV2-23*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --t ---


                    __   CDR1 - IMGT  _____  <------------------------
                               35              40              45
Pr3posvl            ggt ggt tat aac tat ... ... ... gtc tcc tgg tac caa caa cac
Z73664 IGLV2-14*01  --- --- --- --- --- ... ... ... --- --- --- --- --- --g ---
L27822 IGLV2-14*02  --g a-- --- --- ct- ... ... ... --- --- --- --- --- --g ---
Z73665 IGLV2-23*02  --g a-- --- --- ct- ... ... ... --- --- --- --- --- --g ---
Y12412 IGLV2-14*03  --- --- --- --- --- ... ... ... --- --- --- --- --- --- ---
D86994 IGLV2-23*03  --g a-- --- --- ct- ... ... ... --- --- --- --- --- --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 --- --- ... ...
L27822 IGLV2-14*02  --- --- --- --- --- --- --- --- --- --- --g -g- --- ... ...
Z73665 IGLV2-23*02  --- --- --- --- --- --- --- --- --- --- --g --- --- ... ...
Y12412 IGLV2-14*03  --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
D86994 IGLV2-23*03  --- --- --- --- --- --- --- --- --- --- --g -g- --- ... ...


                     - IMGT  _____  <------------------------------------------
                               65              70              75
Pr3posvl            ... ... ... ... ... aat cgg ccc tca ggg gtt tct ... aat cgc
Z73664 IGLV2-14*01  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
L27822 IGLV2-14*02  ... ... ... ... ... --g --- --- --- --- --- --- ... --- ---
Z73665 IGLV2-23*02  ... ... ... ... ... --g --- --- --- --- --- --- ... --- ---
Y12412 IGLV2-14*03  ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
D86994 IGLV2-23*03  ... ... ... ... ... --g --- --- --- --- --- --- ... --- ---


                    ----------------------------  FR3 - IMGT  ---------------
                               80              85              90
Pr3posvl            ttc tct ggc tcc aag ... ... tct ggc aac acg gcc tcc ctg acc
Z73664 IGLV2-14*01  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
L27822 IGLV2-14*02  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
Z73665 IGLV2-23*02  --- --- --- --- --- ... ... --- --- --- --- --- --- --- --a
Y12412 IGLV2-14*03  --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
D86994 IGLV2-23*03  --- --- --- --- --- ... ... --- --- --- --- --- --- --- --a


                    -------------------------------------------------->  ____
                               95              100             104
Pr3posvl            atc tct ggg ctc cag gct gag gac gag gct gat tat tac tgc agc
Z73664 IGLV2-14*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
L27822 IGLV2-14*02  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Z73665 IGLV2-23*02  --- --- --- --- --- --- --- --- --- --- --- --- --- --- t--
Y12412 IGLV2-14*03  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

```
D86994 IGLV2-23*03          --- --- --- --- --- --- --- --- --- --- --- --- --- --- t--


                            _____  CDR3 - IMGT  _____


Pr3posvl                    tca tat aca agc agc agc act gaa gtg ttc ggc gga ggg acc aag
Z73664 IGLV2-14*01          --- --- --- --- --- --- --- ctc
L27822 IGLV2-14*02          --- --- --- --- --- --- --- ctc
Z73665 IGLV2-23*02          --- --- g-- g-t --t --- --- ttc
Y12412 IGLV2-14*03          --- --- --- --- --- --- --- ctc
D86994 IGLV2-23*03          --- --- g-- g-t --t --- --- ttc




Pr3posvl                    ctg acc gtc ctc gag
Z73664 IGLV2-14*01
L27822 IGLV2-14*02
Z73665 IGLV2-23*02
Y12412 IGLV2-14*03
D86994 IGLV2-23*03
```

After we added the missing codons and added the restriction enzyme recognition sites we cloned the light and heavy chain DNA segments into the Pces1 vector as described in "Materials and methods".

We then proceeded to produce Fab Fragments as described in Methods which yielded the following protein quantities:

Table 7: Fab Fragments and their yield per liter of inoculated bacterial culture.

Fab-Fragment	Quantity per liter of bacterial culture
PR3Pos	1 mg
Scu68	1 mg
St140	Negligible

As can be seen from table 7, both the Fab-Fragment PR3-Pos and Scu68 could be produced in a quantity of 1 mg per liter of culture inoculated with the plasmid containing bacteria as described in Methods under "Production of Fabs using the Pces1 Vector". The St140 Fab could not be produced in any significant quantities. We therefore continued integrity testing with the PR3pos and Scu68 Fab fragments.

4.2 Integrity testing of Fab Fragments using SDS-PAGE

The Scu68 and PR3pos Fab fragments underwent denaturation under both reducing and non-reducing conditions and underwent separation based on molecular weight using the method of SDS-PAGE as described unter "Methods". Also, a protein mixture with proteins of known molecular weight was loaded onto the polyacrylamid gel as a reference "ladder". The results are shown in the figure below.
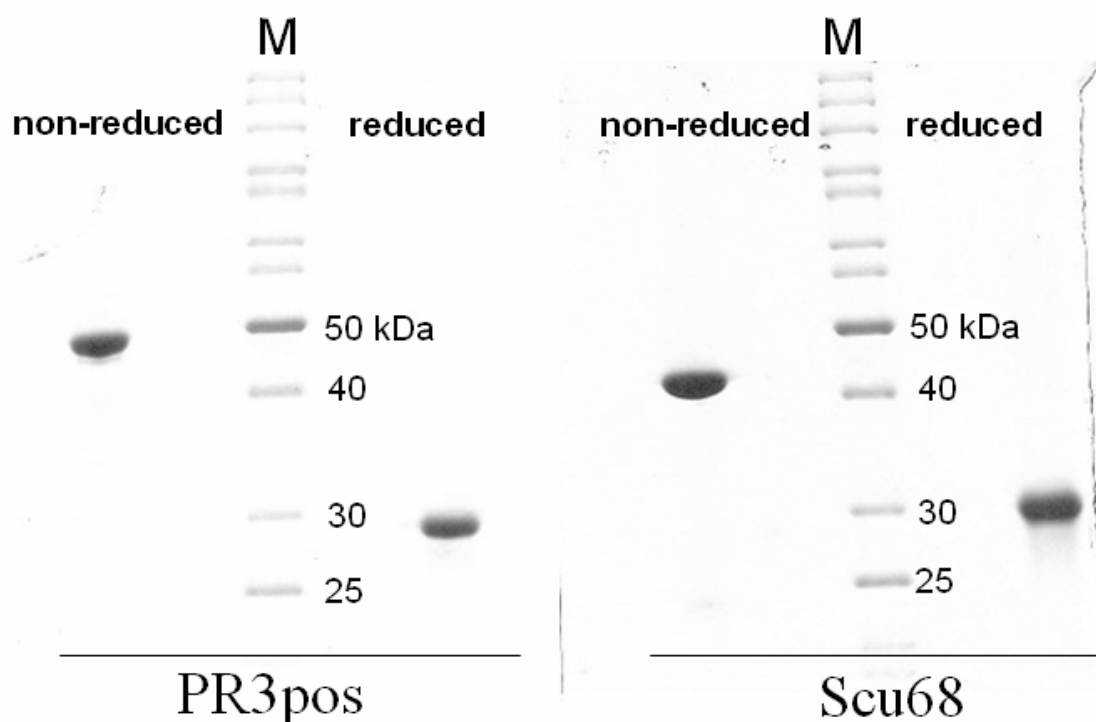
Figure 31:

Results of SDS-PAGE analysis after staining with Coomassie

We can see in figure 31 that both the PR3pos and Scu68 Fab fragments in reduced and unreduced form were loaded onto the polyacrylamid gel and separated by electrophoresis. Thereafter the gel was stained using Coomassie blue. In non-reduced form the Fab migrated in the gel over a distance corresponding to a molecular mass of approximately 50 kDa. In the reduced form, where the disulfide bonds which connect heavy and light chain have been dissolved, a band of approximately 30 kDa appeared. Since heavy and light chain in a Fab fragment are of comparable size only a single band appears under reducing conditions. The

pattern which appears here is consistent with the pattern produced by Fab fragments with known integrity and functionality used in our laboratory. This proves that our Fab Fragments are intact and, thus, that the construction of Fab fragments through cloning is possible.

4.3 Western blotting

To further test the integrity of the Fab Fragments we performed Western blotting as described in "Methods". The proteins were transferred to a PVDF membrane using the semi-dry method. We then incubated with the mouse anti-Penta-His IgG antibody followed by goat anti-mouse IgG antibody (peroxidase coupled) which is a cascade specific for the heavy-chain. Separately we also incubated with the rabbit anti-human-lambda IgG followed by the peroxidase coupled anti-rabbit IgG which is a cascade specific for the light-chain, since both Fab fragments tested here are of the λ variety. After incubation with the peroxidase substrate the following result was obtained.
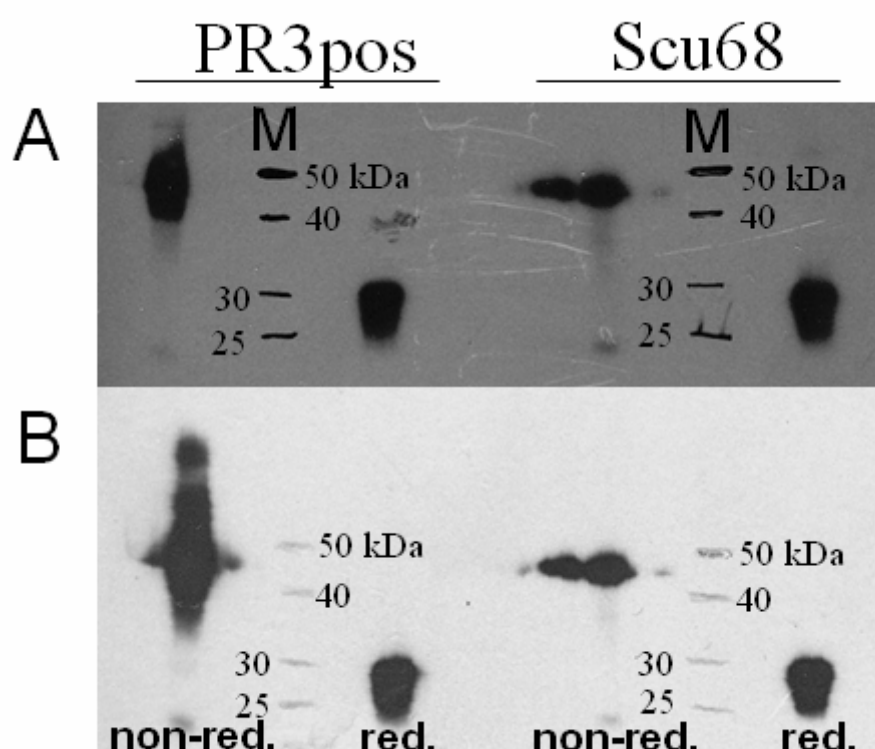

Figure 32:

Western Blot after incubation with the heavy chain cascade, see text

In figure 32 the result of our Western blot is shown for both the denatured samples, shown here as non-reduced and for the samples which, in addition to denaturation through incubation with SDS gel-loading buffer were also reduced, thereby breaking the disulfide bridges. Under non-reducing conditions we saw a band at about 50 kDa, consistent with the known size of a non-reduced Fab fragment, where light and heavy chain are still together. Under reducing conditions the light and heavy chain are no longer held together, but since they are of comparable size still only one band appeared in the Western blot under reducing conditions, as shown in the image. This one band contained both light and heavy chains. In the figure above the result of Western blotting with the heavy chain cascade is shown. The results of Western blotting with the light chain cascade was exactly identical because light and heavy chain are of similar molecular weight.

The Western blot further proves that our Fab fragment was intact. The antibodies used reacted with the His-Tag, which is located in the heavy chain and they reacted with an anti-lambda antibody which is specific for human λ light chain.

4.4 ELISA testing

To check if the coating was done correctly we used the antibody positive controls and a serum positive for PR3-ANCAs of a commercially available PR3-ANCA kit to perform an ELISA using a PR3 coated plate.
The results are shown here.
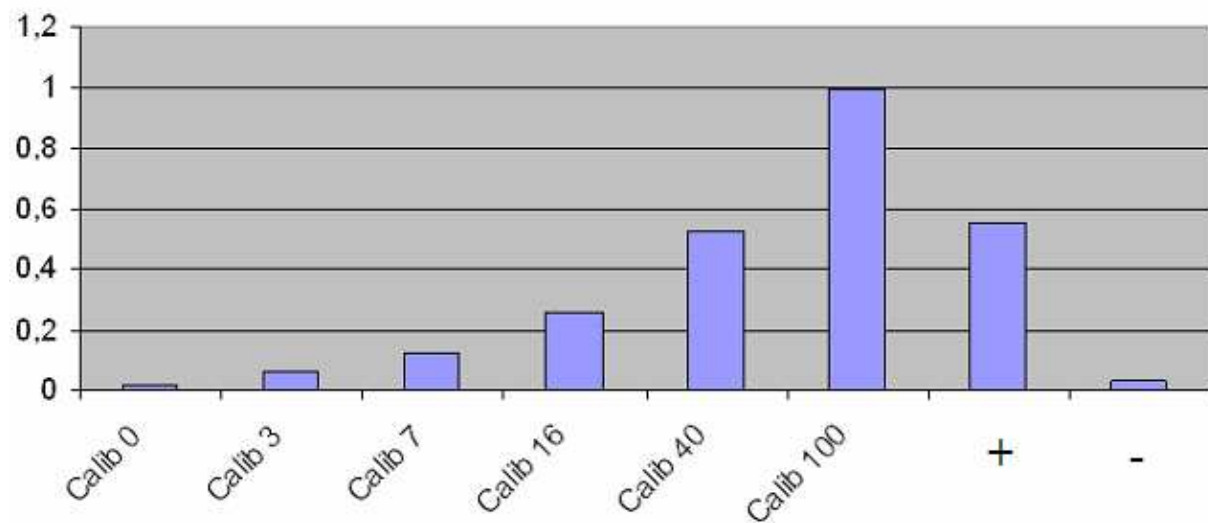
Figure 33:

ELISA results using a commercially available kit with our PR3-coated ELISA plate. X-Axis: various probes, see text. Y-Axis: OD_{450}

The commercially available kit contains an antibody of unknown origin specific for PR3 in increasing concentrations, labelled "Calib" 0-100 here. It also contains a human serum containing an ANCA with specificity for PR3, labelled "+" here and a human serum which serves as a negative control, labelled "-" here. The Calib 0-100 probes serve as a calibration tool for the test and in this case it proved that our PR3-protein was intact and that it reacted with the positive controls of the commercially available kit.

Thereafter we performed an ELISA as described under "methods" using a coating of PR3-protein and our PR3Pos and Scu68 fabs as primary antibodies. The antibody whose DNA-sequence was used to make our PR3pos fab fragment was isolated from a bacterial culture producing this antibody and was also used in the ELISA. This antibody is called WGH1 below.
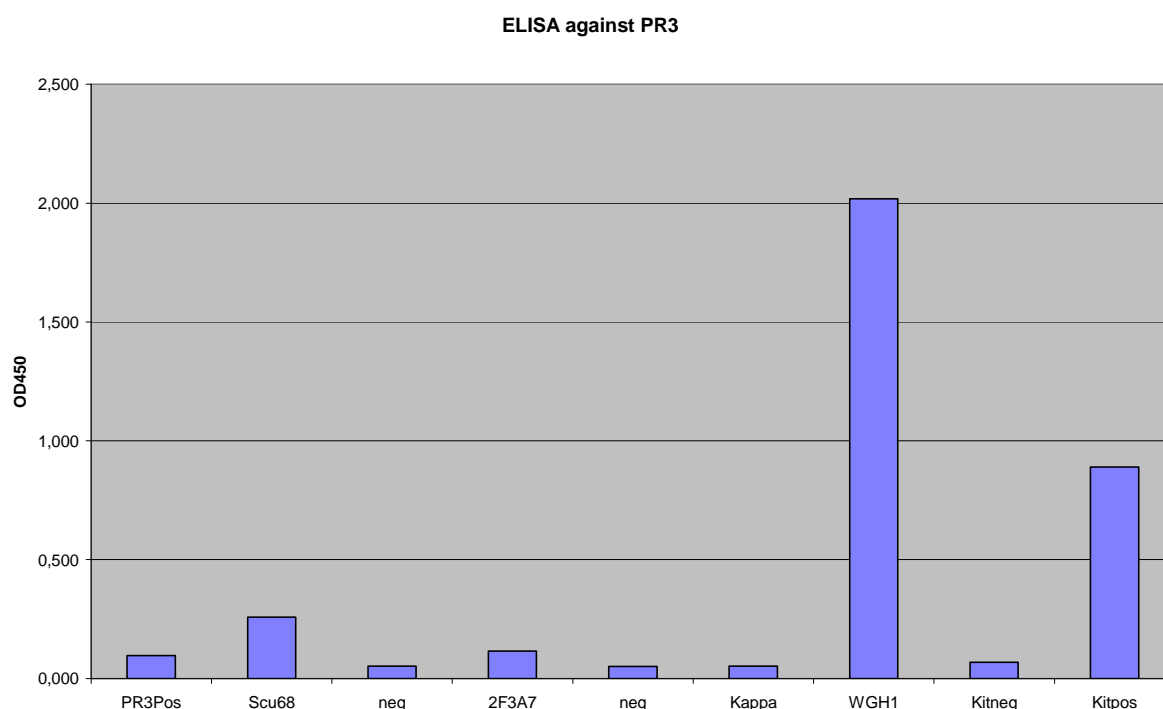
Figure 34:

Results of ELISA testing against a PR3 coating using various antibodies and fab fragments

We used the PR3Pos fab fragment in a concentration of 10 µg/ml, the Scu68 Fab fragment in a concentration of 16 µg/ml. As a negative control we used a lambda Fab fragment with specificity for the influenza matrix protein in an MHC complex, this Fab fragment is called 2F3A7 in the figure above. As a second negative control we used a Fab fragment with the same IMP-MHC specificity which was of the Kappa variety, this Fab fragment is designated "Kappa" in the figure above. The WGH1 antibody is called WGH1 in the figure above and is a full antibody. Additionally we used the positive and negative controls from the commercially available kit used for ANCA testing in the routine patient laboratory, called "Kitpos" and "Kitneg" in the figure above. We also performed an ELISA without any antibody, incubating only with PBS in 1% BSA, this test is called "neg" in the figure above.

As can be seen from the figure above the WGH1 antibody reacts to the PR3 coating. Also the positive control from the commercial kit reacts to the PR3 coating. The PR3Pos Fab fragment does not react against the PR3 protein. The Scu68 fab fragment also does not react against the PR3 protein, although the signal is somewhat higher which might be due to the higher concentration which was used, representing aspecific binding.

This proves that also in this ELISA the coating with PR3 was successful and the PR3 protein was intact after coating. The WGH1 antibody displays specificity for the PR3 protein, as expected. Our PR3Pos Fab fragment does not bind to PR3 protein, even though the DNA sequence used for producing this Fab fragment was identical to the DNA sequence of the WGH1 antibody, with proven specificity for PR3.

5. Discussion

As has been described above, Wegener's granulomatosis basically consists of two entities. The granulomatous lesions mainly occur in the respiratory tract. Often the granulomatous disease is followed by a systemic small-vessel vasculitis which probably is caused by antibodies against PR3. There is a clear chronological order in which these two entities occur. This chronology is the strongest argument for Fienberg's hypothesis, which states that the granulomas eventually give rise to the systemic vasculitis. Opponents of this theory might argue that the early nidi of necrosis out of which the granulomas arise are themselves a consequence of a systemic vasculitis.

Evidence for Fienberg's hypothesis has been accumulating in recent years[33]. B-cell clusters have been observed in the granulomatous lesions found in Wegener's granulomatosis and DNA-analysis of B-cells found in these clusters has shown clonal expansion which is indicative of a germinal center where antigen-driven affinity maturation takes place and therefore represents ectopic lymphoid tissue. On the other hand it is known that the cANCAs are central in the pathogenesis of the systemic vasculitis seen in Wegener's granulomatosis. The B-cells which produce these ANCAs underwent affinity maturation somewhere because they are of the IgG type and it also known that PR3 is found in the vicinity of the B-cell clusters in the granulomatous lesion[33].

Our hypothesis is that formation of the cANCA producing B-cells takes places within the granulomatous lesions seen in Wegener's granulomatosis and that PR3 is the antigen on which this affinity maturation takes place.

Our goal was to produce antibodies, which are similar to the B-cell receptor from antigen (e.g. PR3) – selected B-Lymphocytes that had been isolated from granulomatous WG tissues in order to determine their specificity. We started out with DNA material isolated from individual B cells. First we determined the exact sequence of the DNA material which was present in our samples. In multiple rounds of PCR the variable heavy and light chain coding genes were amplified. We cloned these PCR products into the TOPO-vector which allowed sequencing. The results of sequencing showed that most DNA coding for the heavy chains were lacking the 5' beginning of the framework region. We fixed this problem by supplementing these DNA-fragments with DNA-segments homologous to the respective

germline gene. Furthermore, we added the restriction enzyme recognition sites which were needed to clone the the DNA into the Pces1 vector. This meant adding more nucleotides than is possible in one PCR reaction. In fact, we needed up to four PCR reactions in some cases. As a new approach we mixed the different primers together and performed the PCR reaction in one step. This yielded very mixed results. With some products we could indeed add all nucleotides in one step and with some others the final primer did not bind or the entire reaction did not succeed. If the PCR was not successful the process had to be repeated step by step. Since the success ratio of mixing the primers is not very high the reduction in time and labour was only limited.

In the end we succeeded in cloning all of the DNA coding for the variable domain of the heavy and light chains into the pCes1 vector. This allowed us to produce Fab fragments.

This method represents a totally new approach in handling Fab fragments. Up to now the pCes1 vector was used to mass produce an almost endless array of Fab fragments of different specificities with the goal of isolating a Fab fragment with *known* specificity using the following method. First random DNA sequences coding for heavy and light chains are mass cloned into the pCES1 vector and then these vectors are mass transformed into the appropriate bacterial cultures. The Fab fragments were expressed as a fusion protein connected to a complete phage, which contained the pCes1 vector and therefore the DNA coding for the Fab Fragment. In this way a solid link between the recombinant protein and the DNA coding for this recombinant protein was achieved. The collection of phage with many different random Fab fragments on their surface is an example of a phage library. The Fab fragments have a multitude of different specificities. Using this phage library a series of ELISAs are performed where the phage are first allowed to bind to a selected protein through the Fab fragment which they express on their surface, after which non-binding phage are washed away. Binding phage were eluted and amplified in E. Coli for the next round of ELISA. In the end phage expressing Fab fragments with specificity for the protein used in the ELISA can be derived. The phage library in this case was a random phage library expressing Fab fragments which have either a random specificity or are dysfunctional. Through multiple rounds of ELISAs the derivation of Fab fragments with specificity for the protein used to coat the ELISA plates was possible. Phage libraries can also be used in other experiments, not involving Fab fragments. In another method, a phage library which is derived from the genome of a certain bacterial or mammalian genome, a socalled "natural" peptide library, which is constructed by cleaving

DNA from the appropriate species into the appropriate size and mass cloning the DNA fragments into the pCes1 vector, can be used to present a very wide array of proteins to an antibody of unknown specificity. Through ELISA testing the specificity of this antibody can then be determined. It should be noted, however, that glycosylation, which takes places frequently in mammalian proteins, is not performed using the pCES1 Vector system, since it is a bacterial system, in which glycosylation does not take place. Of course, a random phage library can also contain random proteins, as well as randomly created Fab fragments, which just depends on what is cloned into the pCES-1 vector. These are two different implementations of the phage diplay technology. In the first a library of Fab fragments with different specificities is produced, yielding Fab fragments of known specificity after a process called 'panning' against a known protein. In the second implementation the phage display technology is used to present a specific antibody with a vast array of proteins, either randomly generated or, as in a natural library, derived from the genome of bacteria or mammals. Through the first method described above, Fab fragments are *selected* based upon binding affinity to the protein under investigation. The phage and Fab fragments obtained through this procedure display a proven integrity, otherwise they could not have been selected through binding in the ELISA.

Our approach was different and new. We have not obtained our Fab fragment through selection, which guarantees binding capability and integrity. We have *constructed* our Fab fragments on the basis of DNA information derived from a B-cell coding for a full antibody and on the basis of DNA information derived from a hybridoma producing an antibody with known specifity for PR3. We could not assume that our Fab fragments are structurally intact because the way in which they were produced is very different from the selection described above. Of note, our Fab fragments were constructed based upon information from single B cells and consecutively are of unknown specificity, which is another difference from the method described above. The aim of our study was to investigate the specifity of B cell receptors found in the granulomatous lesions found in Wegener's granulomatosis patients. As a proof-of-principle we first investigated the structural integrity of the Fab fragments we produced. As a second step we investigated the binding capacity of our positive control, called PR3pos, to the PR3 protein, using ELISA.

The first step was necessary because our Fab fragment was a hybrid; The DNA coding for the variable regions was derived from our B cells or antibody-producing hybridoma cell line while the constant regions were in the pCES-1 vector itself. Therefore it was not certain that

this hybrid still had the same structure as as Fab fragment, since there are many transitional regions in the DNA coding for an antibody as a result of VDJ rearrangement. These transitional regions might also be involved in protein folding. The second step was necessary to investigate if the intact Fab fragment retains the same specificity as the full antibody it was derived from. This is not certain. The Fab fragment is similar to a full antibody, but the Fc fragment is missing, which might cause a conformational change and change of specificity. The same is true for the Fab fragments which are derived using selection as described above. These Fab fragments might not bind to the protein upon which they were selected if they were produced as full antibodies. In order to answer the question of retention of specificity when removing or adding the Fc-fragment it is very important to have a positive control based on an antibody with known specificity. For this purpose we set out to construct a Fab fragment with specificity for the PR3 protein. We isolated the DNA from a hybridoma which produces an anti-PR3 antibody. We cloned this DNA into our Pces1 vector just like we did for the DNA which was isolated from the B-cells. The DNA sequence of the DNA we derived from the hybridoma was exactly identical to the DNA sequence which was described in a paper[3] about a PR3-specific antibody, derived from a Wegeners granulomatosis patient, called WGH1.

We proved the integrity of our Fab fragments by two different methods. In a first step we used SDS-PAGE to investigate the molecular weight of the proteins we produced. The molecular weight we found was comparable to Fab fragments with known integrity and proven binding capability. Under reducing circumstances the bands in our gel were found at a level corresponding to a protein size of about 30 kDa, which is consistent with the results of Fab fragments with known integrity. We conclude that our protein consist of two parts which can be separated under circumstances which break up disulfide bridges. These two parts probably represent heavy and light chain. To further prove this idea we used Western blotting with immunological probing by two different blotting approaches. We used two different development cascades in our Western blots. One was specific for His-Tag known to be present in the heavy chain of Fab Fragments produced using the pCES-1 vector as used in our experiments and the other cascade was very specific for the human lambda light chain. The results were identical to results derived from Western blotting using Fab fragments with known integrity and binding capability. The antibodies bound specifically to heavy and light chain which in the end proves the integrity of our Fab fragments definitively.

Regarding the question of preservation of specificity when producing a Fab fragment based on information derived from a full antibody we performed an ELISA using the PR3 protein. In a first step we proved that the coating of the ELISA plates using our PR3 was successful using a commercially available kit used for determination of the ANCA-titer in the clinical laboratory. After that we used our PR3pos Fab fragment to test for specificity for the PR3 protein. Additionally we used our Scu68 Fab fragment to test for specificity against PR3. As shown in the last figure in the Results chapter our PR3Pos fab fragment did not bind to PR3. At the same time the full antibody from which the DNA sequence was derived to produce this fab fragment did bind to PR3. This proves that the DNA sequence is coding for a PR3 binding antibody. Above, we also showed that our fab fragments are structurally intact and we checked that we correctly cloned the DNA coding for the variable regions of the WGH1 antibody into our Pces1 vector. Therefore, it is certain that we have an intact fab fragment with a protein sequence identical to the protein sequence of the variable part of the WGH1 antibody which binds PR3 protein. Still our fab fragment does not bind PR3. The most probable explanation for the Fabs non-affinity to PR3 is a conformational change due to the lack of a Fc-receptor or a different tertiary structure in the Fab fragment.

A review of PR3-binding antibodies was given by E. Peen and R.C. Williams jr[21]. In a table derived from this review we hereby show the aminoacid sequence of some PR3-binding antibodies, data derived from Davis[3] and Sibilia[28].

Table 8: PR3-binding antibodies and their characteristics.

Designation	VH CDR3 sequence	VL CDR3 sequence
WGH1[3]	SQMSYY**D**FWSGYYR**D**QYY	SSYTSSST**E**V
WG1[28]	VAVAGGF**D**PWGQGTLVT	
WG2[28]	GE**ID**FWSGYFR**E**GLVGP	**ED**FATYYCLQHNSYP
WG3[28]	RGIF**E**GSGYYAL**DD**WGQG	QQYYGTPFTFGPGTKV**D**IKR
WG4[28]	SRGTSCYMSC**D**CWGPRTL	QQHYGTPFTFGTKV**D**IKR

The WGH1 is the sequence of our pr3pos-fab. As reviewed in Peen and Williams[21] the negatively charged glutamic acids (E) and aspartic acids (D) are multiply present in the aminoacid sequence. This is consistent with the findings of our group, Voswinkel et al[31,33] who found a skewing towards negatively charged aminoacids in the immunoglobulin coding sequences found in granulomatous formations in WG patients. It is evident that there is

considerable variation in the aminoacid sequence of published PR3-binding antibodies. The PR3-antibody we used to produce our Fab had less negatively charged aminoacids and is therefore a little different from the other PR3-antibodies. Comparative studies investigating the affinity of PR3-antibodies have not been published. Further, no quantitative studies analysing the nature of the PR3-binding antibodies in sera from WG-patients have been performed.

Our method represents a new approach in handling Fab fragments. Other groups performed screening of millions of randomly formed Fabs displayed on phage against the protein of interest using an ELISA plate with washing steps. Only those phage displaying Fabs that bind to the coated protein are used to perform further studies. In this situation two things are known. First, the Fab fragments are functional and bind to a certain protein. Secondly, the protein to which the Fab fragments binds is known. We had a completely different approach: we isolated DNA coding from whole antibodies and used this DNA to construct Fab fragments. We did not know the specificity of these Fab fragments and because we constructed these fragments instead of selecting them out of a library using ELISAs we also did not know if these Fab fragments are functional. Put simply, a Fab fragment is an antibody without the Fc fragment. Because the Fc fragment is lacking, the variable domain might have a slightly different folding or orientation which might alter the specificity or disable functionality as a whole. This possibility cannot be excluded and might explain the inability of the PR3Pos fab to bind the PR3 protein. At the moment our group is developing a system to produce whole antibodies instead of Fab fragments with the intention of repeating ELISA screening and performing phage library screening. By producing whole antibodies we get closer to the human disease in which ANCAs typically are IgG antibodies.

In summary, we succeeded in preparing DNA derived from B cells from granulomatous lesions of Wegeners patients and successfully used this DNA to produce intact Fab fragments with a variable region protein sequence which is identical with the antibody or B-cell receptor produced by the respective single B cell from which the DNA was derived. Either due to conformational changes caused by the removal of the Fc Fragment in a Fab fragment or due to a different tertiary structure of the Fabs as compared to the whole antibody our fabs failed to bind a protein target. Another explanation is that the specificity of the Fab fragment has changed due to the replacement of nucleotides which were lost in the PCR reactions

performed after capture of single B cells with germline sequence, although this is unlikely since the highly variable complementary determining regions were preserved.

Perspectives

As described above, further work is currently being undertaken to produce full antibodies instead of mere Fab fragments which will presumably answer this question. This study laid the groundwork for these efforts, which will help to investigate the nature of the B cell receptors found in granulamatous lesions of Wegeners patients and elucidate their precise role. It is possible that our hypothesis, which states that the anti-PR3 antibody arises in the granulomas found in the respiratory tract, will be confirmed, which will lead to a profound understanding of the still rather unclear pathophysiologic mechanisms leading to this disease. On the other hand, the characterization of the specificity of the B cell receptors found in the respiratory tract lesions might lead to the identification of other antigens which might play a role in pathophysiology. There is, for instance, no clear explanation for the protective effect of cotrimoxazol treatment. Treatment with co-trimoxazole leads to a significant reduction in respiratory tract and other infections and leads to a significant reduction in the incidence of relapse in WG patients who are in remission. At the same time there is an association between chronic nasal carriage of Staph. aureus and relapse rates in WG. If the B cell receptors would turn out to be specific for parts of the Staph. aureus bacterium these findings could be better understood. Possibly, other pathogenic agents might be identified. Whatever the target of the B cells found in the granulamatous lesions of WG patients might be, it is clear that its characterization will lead to a much better understanding of the underlying pathophysiology and open the road to new therapeutic possibilities. As an example, once pivotal antigens in the genesis of granulomatous lesions found in Wegeners granulomatosis have been identified, blocking these antigens with an in vitro produced antibody (analogous to the anti CD-20 antibody known as Rituximab) would deprive the B cells found in the granulomatous lesions of stimulation, possibly leading to an interruption of the inflammatory process and inducement of a remission.

Appendix I: Results of sequencing

St140 VH

```
                         <---------------------------------------- FR1 - IMGT
                         1               5               10              15
st140vh                  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L10088 IGHV4-59*01       cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
M29812 IGHV4-59*02       cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
X56360 IGHV4-59*07       cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
M95114 IGHV4-59*03       cag gtg cag ctg cag gag tcg ggc cca ... gga ctg gtg aag cct
X87091 IGHV4-59*08       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...


                         ---------------------------------------->  _____
                         20              25              30
st140vh                  ... ... ..c ctg tcc ctc acc tgc gct gtc tct ggt gac tcc ctc
L10088 IGHV4-59*01       tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
M29812 IGHV4-59*02       tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- g--
X56360 IGHV4-59*07       tcg gac ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
M95114 IGHV4-59*03       tcg gag ac- --- --- --- --- --- a-- --- --- --- -g- --- a--
X87091 IGHV4-59*08       ... ... ... ... --- --- --- --- a-- --- --- --- -g- --- a--


                         __   CDR1 - IMGT _____  <------------------------
                                          35              40              45
st140vh                  ggt agt tac tac ... ... ... ... tgt acc tgg atc cga cag ccc
L10088 IGHV4-59*01       a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
M29812 IGHV4-59*02       a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
X56360 IGHV4-59*07       a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
M95114 IGHV4-59*03       a-- --- --- --- ... ... ... ... --g -g- --- --- --g --- ---
X87091 IGHV4-59*08       a-n --- --- --- ... ... ... ... --g -g- --- --- --g --- ---


                         FR2 - IMGT  ------------------------>  _____  CDR2
                                          50              55              60
st140vh                  ccc ggg aag gga ctg gaa tgg gtg ggt tat atc tat tac agt ggg
L10088 IGHV4-59*01       --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
M29812 IGHV4-59*02       --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
X56360 IGHV4-59*07       --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
M95114 IGHV4-59*03       --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---
X87091 IGHV4-59*08       --a --- --- --- --- --g --- a-t --g --- --- --- --- --- ---


                         - IMGT _____  <------------------------------------
                                          65              70              75
st140vh                  agc acc ... ... ... aat tat aac cct tcc ctc aag ... agt cga
L10088 IGHV4-59*01       --- --- ... ... ... --c --c --- --c --- --- --- ... --- ---
M29812 IGHV4-59*02       --- --- ... ... ... --c --c --- --c --- --- --- ... --- ---
X56360 IGHV4-59*07       --- --- ... ... ... --c --c --- --c --- --- --- ... --- ---
M95114 IGHV4-59*03       --- --- ... ... ... --c --c --- --c --- --- --- ... --- ---
X87091 IGHV4-59*08       --- --- ... ... ... --c --c --- --c --- --- --- ... --- ---


                         ------------------------------ FR3 - IMGT  --------------
```

```
                                           80              85              90
st140vh                    gcc gtc ata tca atc gac aca acc aac aac cgg ttc tcc ctg aac
L10088 IGHV4-59*01         -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
M29812 IGHV4-59*02         -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
X56360 IGHV4-59*07         -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g
M95114 IGHV4-59*03         -t- ac- --- --- g-a --- --g t-- --g --- -aa --- --- --- --g
X87091 IGHV4-59*08         -t- ac- --- --- g-a --- --g t-- --g --- -a- --- --- --- --g


                           --------------------------------------------------------> ____
                                           95              100             104
st140vh                    ctc agc tct gtg acc gct gga gac acg gcc gtg tat tat tgt gtg
L10088 IGHV4-59*01         --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
M29812 IGHV4-59*02         --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
X56360 IGHV4-59*07         --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
M95114 IGHV4-59*03         --g --- --- --- --- --- -cg --- --- --- --- --- --c --- -c-
X87091 IGHV4-59*08         --g --- --- --- --- --c -c- --- n-- --- --- --- --c --- -c-


                           _____    CDR3 -  IMGT _____

st140vh                    aga gga aac ggc gcg ctc cta tcc tgg ggc caa gga acc ctg gt
L10088 IGHV4-59*01         --- -a-
M29812 IGHV4-59*02         --- -a-
X56360 IGHV4-59*07         ---
M95114 IGHV4-59*03
X87091 IGHV4-59*08         ---
```

St140vκ

```
                           <--------------------------------------------- FR1 - IMGT
                           1          5              10              15
st140vk                    ... ... ... ... ... cag tct cca tct tcc gtg tct gca tct gta
V01577 IGKV1-12*01         gac atc cag atg acc --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02         gac atc cag atg acc --- --- acc --- --- --- --- --- --- ---
X17263 IGKV1D-12*01        gac atc cag atg acc --- --- --- --- --t --- --- --- --- ---
K01323 IGKV1D-16*01        gac atc cag atg acc --- --- --- --c --a c-- --- --- --- ---
V00558 IGKV1D-16*02        gac atc cag atg acc --- --- --- --c --a c-- --- --- --- ---


                           ----------------------------------------> _____
                                        20              25              30
st140vk                    gga gac aga gtc acc atc act tgt cgg gcg agt cag ggt att agc
V01577 IGKV1-12*01         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02         --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01        --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
K01323 IGKV1D-16*01        --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V00558 IGKV1D-16*02        --- --- --- --- --- --- --- --- --- --- --g --- --- --- ---


                           __  CDR1 - IMGT  _____ <------------------------
                                        35              40              45
st140vk                    agc tgg ... ... ... ... ... ... tta gcc tgg tat cag cag aaa
V01577 IGKV1-12*01         --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
V01576 IGKV1-12*02         --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
X17263 IGKV1D-12*01        --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
```

```
K01323 IGKV1D-16*01          --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---
V00558 IGKV1D-16*02          --- --- ... ... ... ... ... ... --- --- --- --- --- --- ---


                    FR2 - IMGT ------------------------->  _____ CDR2
                                     50              55              60
st140vk                      cca ggg aaa gcc cct aag ctc ctg atc tat gct gca tcc ... ...
V01577 IGKV1-12*01           --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
V01576 IGKV1-12*02           --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
X17263 IGKV1D-12*01          --- --- --- --- --- --- --- --- --- --- --- --- --- ... ...
K01323 IGKV1D-16*01          --- -a- --- --- --- --- tc- --- --- --- --- --- --- ... ...
V00558 IGKV1D-16*02          --- -a- --- --- --- --- tc- --- --- --- --- --- --- ... ...


                    - IMGT _____ <-------------------------------------
                                     65              70              75
st140vk                      ... ... ... ... ... agt ttg caa agt ggg gtc cca ... tca agg
V01577 IGKV1-12*01           ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
V01576 IGKV1-12*02           ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
X17263 IGKV1D-12*01          ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
K01323 IGKV1D-16*01          ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---
V00558 IGKV1D-16*02          ... ... ... ... ... --- --- --- --- --- --- --- ... --- ---


                    ---------------------------- FR3 - IMGT  --------------
                                     80              85              90
st140vk                      ttc agc ggc agt gga ... ... tct ggg aca gat ttc act ctc acc
V01577 IGKV1-12*01           --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02           --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01          --- --- --- --- --- ... ... --- --- --- --- --- --- --- --t
K01323 IGKV1D-16*01          --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---
V00558 IGKV1D-16*02          --- --- --- --- --- ... ... --- --- --- --- --- --- --- ---


                    ------------------------------------------------------> ____
                                     95              100             104
st140vk                      atc agc agc ctg cag cct gaa gat ttt gca act tac tat tgt caa
V01577 IGKV1-12*01           --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
V01576 IGKV1-12*02           --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
X17263 IGKV1D-12*01          --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
K01323 IGKV1D-16*01          --- --- --- --- --- --- --- --- --- --- --- --t --c --c ---
V00558 IGKV1D-16*02          --- --- --- --- --- --- --- --- --- --- --- --t --c --c ---


                    _____ CDR3 - IMGT _____

st140vk                      cag gct aac agt ttc cct cca agc acc ttc ggc caa ggg aca cg
V01577 IGKV1-12*01           --- --- --- --- --- --- --
V01576 IGKV1-12*02           --- --- --- --- --- --- t-
X17263 IGKV1D-12*01          --- --- --- --- --- --- --
K01323 IGKV1D-16*01          --- ta- --t --- -a- --- --
V00558 IGKV1D-16*02          --- ta- --t --- -a- --- --
```

107

Scu68vh

```
                    <------------------------------------------ FR1 - IMGT
                    1             5             10            15
scu68vh             ... ... ... ... ... ... ..g ggg acc ... agg gtt cct tgg ccc
M29811 IGHV4-61*01  cag gtg cag ctg cag gag tc- --c c-a ... g-a c-g gtg aa- --t
X92230 IGHV4-61*03  cag gtg cag ctg cag gag tc- --c c-a ... g-a c-g gtg aa- --t
AB019437 IGHV4-61*08 cag gtg cag ctg cag gag tc- --c c-a ... g-a c-g gtg aa- --t
M29812 IGHV4-59*02  cag gtg cag ctg cag gag tc- --c c-a ... g-a c-g gtg aa- --t
X56356 IGHV4-61*05  cag ctg cag ctg cag gag tc- --c c-a ... g-a c-g gtg aa- --t


                    ------------------------------------------> _____
                    20            25            30
scu68vh             cgg gtg acc ctg tcc ctc acc tgc act gtc tct gga gtc tcc gtc
M29811 IGHV4-61*01  tc- -a- --- --- --- --- --- --- --- --- --- --t -g- --- ---
X92230 IGHV4-61*03  tc- -a- --- --- --- --- --- --- --- --- --- --t -g- --- ---
AB019437 IGHV4-61*08 tc- -a- --- --- --- --- --- --- --- --- --- --t -g- --- ---
M29812 IGHV4-59*02  tc- -a- --- --- --- --- --- --- --- --- --- --t -g- --- ---
X56356 IGHV4-61*05  tc- -a- --- --- --- --- --- --- --- --- --- --t -g- --- a--


                    __  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--
X92230 IGHV4-61*03  agc ag- -g- -g- --c --c ... ... --- -g- --- --c --g --- c--
AB019437 IGHV4-61*08 agc ag- -g- gg- --c --c ... ... --- -g- --- --c --g --- c--
M29812 IGHV4-59*02  agt ag- t-c tac ... ... ... ... --- -g- --- --c --g --- c--
X56356 IGHV4-61*05  agc ag- ag- -g- --c --c ... ... --- gg- --- --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 ---
X92230 IGHV4-61*03  --- --- --- --- --- --- --- --- --g t-- a-- --- --- --t ---
AB019437 IGHV4-61*08 --- --- --- --- --- --- --- --- --g t-- a-- --- --- --t ---
M29812 IGHV4-59*02  --- --- --- --- --- --- --- --- --g t-- a-- --- --- --t ---
X56356 IGHV4-61*05  --- --- --- --- --- --- --- --- --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-- ... --- ---
X92230 IGHV4-61*03  -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
AB019437 IGHV4-61*08 -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
M29812 IGHV4-59*02  -gc -c- ... ... ... a-- --- --- --- --- --- a-- ... --- ---
X56356 IGHV4-61*05  -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
X92230 IGHV4-61*03  --- --- a-a --a g-a --- --- --- --g --c --c --- --c --- --g
AB019437 IGHV4-61*08 --- --- a-a --a g-a --- --- --- --g --c --- --- --c --- --g
```

108

```
M29812 IGHV4-59*02          --- --- a-a --a g-a --- --- --- --g --c --- --- --c --- --g
X56356 IGHV4-61*05          --- --- a-a --a g-a --- -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-
X92230 IGHV4-61*03          --- --c --- --- --- g-- --- --- --- --- g-g --- -ac --- -c-
AB019437 IGHV4-61*08        --- --c --- --- --- g-- --- --- --- --- g-g --- -ac --- -c-
M29812 IGHV4-59*02          --- --c --- --- --- g-- --- --- --- --- g-g --- -ac --- -c-
X56356 IGHV4-61*05          --- --c --- --- --- g-c --- --- --- --- 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          --- --
X92230 IGHV4-61*03          --- --
AB019437 IGHV4-61*08        --- --
M29812 IGHV4-59*02          --- --
X56356 IGHV4-61*05          ---


                                     ___


scu68vh                     ccc tgg ggc caa gga ccc ctg gtc aca
M29811 IGHV4-61*01
X92230 IGHV4-61*03
AB019437 IGHV4-61*08
M29812 IGHV4-59*02
X56356 IGHV4-61*05
```

Scu68vl

```
                            <------------------------------------------ FR1 - IMGT
                            1            5                  10                  15
scu68vl                     tct tct gag ctg act cag gac cct gct ... gtg tct gtg gcc ttg
X56178 IGLV3-19*01          --- --- --- --- --- --- --- --- --- ... --- --- --- --- ---
Z73645 IGLV3-32*01          --c --- -g- -ca --- --- -tg --- --a ... --- --- --- --- ---
X57826 IGLV3-1*01           --c -a- --- --- --- --- cca --c t-a ... --- --c --- t-- cca
X97473 IGLV3-9*01           --c -a- --- --- --- --- cca -tc t-a ... --- --a --- --- c--
D86994 IGLV3-27*01          --c -a- --- --- --a --- cca t-c t-a ... --- --a --- t-t cc-


                            ----------------------------------------> _____
                                        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
Z73645 IGLV3-32*01          --a --a -tg -c- -gg --- --c --- --- --- --- --- a-g g-- g-c
X57826 IGLV3-1*01           --a --- --- -c- -gc --- --c --- tct --- --t -aa t-g ggg gat
X97473 IGLV3-9*01           --a --- --g -c- -gg --t --c --t gg- --- a-- -a- a-t gg- --t
D86994 IGLV3-27*01          --a --- --- -c- -gg --- --c --- tca --- --t gta --g gc- -aa


                            __ CDR1 - IMGT _____ <-------------------------
                                        35                40                  45
scu68vl                     tat tat ... ... ... ... ... ... gca ggc tgg tat aag cag aag
```

109

```
X56178 IGLV3-19*01          --- --- ... ... ... ... ... ... --- a-- --- --c c-- --- ---
Z73645 IGLV3-32*01          -c- --- ... ... ... ... ... ... -a- ca- --- --c c-- --- ---
X57826 IGLV3-1*01           a-a --- ... ... ... ... ... ... --t t-- --- --- c-- --- ---
X97473 IGLV3-9*01           a-a a-- ... ... ... ... ... ... -tg ca- --- --c c-- --- ---
D86994 IGLV3-27*01          a-a --- ... ... ... ... ... ... --t c-g --- -tc 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- --- --- ... ...
Z73645 IGLV3-32*01          --- --c --- --- --c gtg --g --- --c --- -a- -gc -gt ... ...
X57826 IGLV3-1*01           --- --c --- t-- --- gtg --g --- --c --- caa g-t -g- ... ...
X97473 IGLV3-9*01           --- --c --- --- --- gtg --g --- --c --- agg g-t -g- ... ...
D86994 IGLV3-27*01          --- --c --- --- --- gtg --g --g --- --- aaa g-c -gt ... ...


                       - IMGT  _____  <-------------------------------------
                                  65                70                75
scu68vl                     ... ... ... ... ... agg cgg ccc tca ggg atc cca ... gac cga
X56178 IGLV3-19*01          ... ... ... ... ... -ac --- --- --- --- --- --- ... --- ---
Z73645 IGLV3-32*01          ... ... ... ... ... gac --- --- --- a-- --- --t ... --g ---
X57826 IGLV3-1*01           ... ... ... ... ... -a- --- --- --- --- --- --t ... --g ---
X97473 IGLV3-9*01           ... ... ... ... ... -ac --- --- --t --- --- --t ... --g ---
D86994 IGLV3-27*01          ... ... ... ... ... ga- --- --- --- --- --- --t ... --g ---


                      --------------------------------  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 --- --- ---
Z73645 IGLV3-32*01          --- --- --c --- --a ... ... --- --c a-- --- a-- a-- c-- ---
X57826 IGLV3-1*01           --- --- --c --- --- ... ... --t --g a-- --- --- a-t c-- ---
X97473 IGLV3-9*01           --- --- --c --- --- ... ... --g --g a-- --g --- a-- c-- ---
D86994 IGLV3-27*01          --- --c --c --- -g- ... ... --- --g ac- --- -t- a-- --- ---


                      ----------------------------------------------------->  ____
                                  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
Z73645 IGLV3-32*01          --- --- --- --c --- --t --g --- --- --- --t --- -a- -a- c-g
X57826 IGLV3-1*01           --- -gc --- a-c --- --t atg --- --- --- --c --- -a- --- c-g
X97473 IGLV3-9*01           --- -gc a-a --c --a --c -gg --- --- --- --c --- -a- --- c-g
D86994 IGLV3-27*01          --- -gc --- --c --- -tt --g --- --- --- --c --- -a- --- t-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
Z73645 IGLV3-32*01          -tg ata --- aa- ca- -ct -c
X57826 IGLV3-1*01           g-g t-g --- a-- agc act gca
X97473 IGLV3-9*01           gtg t-g --- a-- agc act gca
D86994 IGLV3-27*01          --t gcg -ct -a- aac a-t ct
```

110

```
scu68vl                          acc aag ctg acc gtc
X56178 IGLV3-19*01
Z73645 IGLV3-32*01
X57826 IGLV3-1*01
X97473 IGLV3-9*01
D86994 IGLV3-27*01
```

Acknowledgement

I was very happy to be able to do translational research and I would like to express my sincere gratitude to Prof. Dr. M. Pfreundschuh for giving me the opportunity. I thank Dr. J. Voswinkel for his wonderful coaching, friendly advice and direct communication during the entire experimental period and writing period thereafter. Also I thank Ms. G. Carbon for introducing me to laboratory techniques and the friendly support and advice during the entire period of experimentation. And I would like to give a big thanks to Ms. B. Bette for her help with the final ELISAs and for being a great colleague.

Then I would like to thank all the people in the José Carreras Center for Immuno- and Gene Therapy of the department of Internal Medicine of University of the Saarland, Germany. You were wonderful colleagues and I really liked the friendly atmosphere in which I worked. I especially thank a number of persons who helped me with my research: Dr. G. Held and Dr. D. Preuss for help with phage library screening and Prof. Dr. K. Roemer and Kristina Heyne for their help with Western blots.

Curriculum vitae

<u>Personal data:</u>

Name:	Augustijn Johannes Kerkdijk
Date of birth;	23.12.1976
Place of birth:	Purmerend, the Netherlands

<u>Education:</u>

1995	High School Diploma, Da Vinci College in Purmerend
1995 – 1996	Study of Physics and Computer Science at University of Amsterdam
1996 – 2002	Study for degree of Master of Science in Medicine
2002 – 2004	Practical period of medical education, followed by medical registration in the Netherlands

<u>Work experience:</u>

2005 – 2006	Department of Internal Medicine of the Prison Hospital of the State of North Rhine-Westphalia, Germany
2006 – 2007	M.D. studies at the University of the Saarland, Germany
2007 - 2008	Department of Hematology and Oncology, University Medical School of the Saarland, Germany
2008 -	Department of Diagnostic and Interventional Radiology, University of Leipzig, Germany

Copyright information and notices

List of references

1. Aries,P.M., Lamprecht,P. & Gross,W.L. Wegener's granulomatosis: a view from the granulomatous side of the disease. *Isr. Med. Assoc. J.* **7**, 768-773 (2005).

2. Bosch,X., Guilabert,A. & Font,J. Antineutrophil cytoplasmic antibodies. *Lancet* **368**, 404-418 (2006).

3. Davis,J.A. *et al.* Determination of primary amino acid sequence and unique three-dimensional structure of WGH1, a monoclonal human IgM antibody with anti-PR3 specificity. *Clin. Immunol. Immunopathol.* **89**, 35-43 (1998).

4. de Haard,H.J. *et al.* A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J. Biol. Chem.* **274**, 18218-18230 (1999).

5. Drayton,D.L., Liao,S., Mounzer,R.H. & Ruddle,N.H. Lymphoid organ development: from ontogeny to neogenesis. *Nat. Immunol.* **7**, 344-353 (2006).

6. Falk,R.J., Terrell,R.S., Charles,L.A. & Jennette,J.C. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc. Natl. Acad. Sci. U. S. A* **87**, 4115-4119 (1990).

7. Fink,L., Kwapiszewska,G., Wilhelm,J. & Bohle,R.M. Laser-microdissection for cell type- and compartment-specific analyses on genomic and proteomic level. *Exp. Toxicol. Pathol.* **57 Suppl 2**, 25-29 (2006).

8. Fujinaga,M., Chernaia,M.M., Halenbeck,R., Koths,K. & James,M.N. The crystal structure of PR3, a neutrophil serine proteinase antigen of Wegener's granulomatosis antibodies. *J. Mol. Biol.* **261**, 267-278 (1996).

9. Hagen,E.C. *et al.* Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. EC/BCR Project for ANCA Assay Standardization. *Kidney Int.* **53**, 743-753 (1998).

10. Hoffman,G.S. *et al.* Wegener granulomatosis: an analysis of 158 patients. *Ann. Intern. Med.* **116**, 488-498 (1992).

11. Invitrogen Corp. Zero Blunt TOPO PCR Cloning Kit manual. (2006).

12. Jennette,J.C. *et al.* Nomenclature of systemic vasculitides. Proposal of an international consensus conference. *Arthritis Rheum.* **37**, 187-192 (1994).

13. Kallenberg,C.G., Heeringa,P. & Stegeman,C.A. Mechanisms of Disease: pathogenesis and treatment of ANCA-associated vasculitides. *Nat. Clin. Pract. Rheumatol.* **2**, 661-670 (2006).

14. Kuppers,R., Zhao,M., Hansmann,M.L. & Rajewsky,K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J.* **12**, 4955-4967 (1993).

15. Lamprecht,P. & Gross,W.L. Wegener's granulomatosis. *Herz* **29**, 47-56 (2004).

16. Lamprecht,P., Trabandt,A. & Gross,W.L. Clinical and immunological aspects of Wegener's granulomatosis (WG) and other syndromes resembling WG. *Isr. Med. Assoc. J.* **2**, 621-626 (2000).

17. Ludemann,J., Utecht,B. & Gross,W.L. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. *J. Exp. Med.* **171**, 357-362 (1990).

18. Mahr,A.D., Neogi,T. & Merkel,P.A. Epidemiology of Wegener's granulomatosis: Lessons from descriptive studies and analyses of genetic and environmental risk determinants. *Clin. Exp. Rheumatol.* **24**, S82-S91 (2006).

19. Mullen,L.M., Nair,S.P., Ward,J.M., Rycroft,A.N. & Henderson,B. Phage display in the study of infectious diseases. *Trends Microbiol.* **14**, 141-147 (2006).

20. Muller,A. *et al.* Localized Wegener's granulomatosis: predominance of CD26 and IFN-gamma expression. *J. Pathol.* **192**, 113-120 (2000).

21. Peen,E. & Williams,R.C., Jr. What you should know about PR3-ANCA. Structural aspects of antibodies to proteinase 3 (PR3). *Arthritis Res.* **2**, 255-259 (2000).

22. Popa,E.R., Stegeman,C.A., Kallenberg,C.G. & Tervaert,J.W. Staphylococcus aureus and Wegener's granulomatosis. *Arthritis Res.* **4**, 77-79 (2002).

23. Radford,D.J., Savage,C.O. & Nash,G.B. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. *Arthritis Rheum.* **43**, 1337-1345 (2000).

24. Rao,N.V. *et al.* Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. *J. Biol. Chem.* **266**, 9540-9548 (1991).

25. Reinhold-Keller,E. *et al.* An interdisciplinary approach to the care of patients with Wegener's granulomatosis: long-term outcome in 155 patients. *Arthritis Rheum.* **43**, 1021-1032 (2000).

26. Sambrook, Fritsch & Maniatis. Molecular Cloning, a laboratory manual second edition. 1-1-1989.

27. Sarraf,P. & Sneller,M.C. Pathogenesis of Wegener's granulomatosis: current concepts. *Expert. Rev. Mol. Med.* **7**, 1-19 (2005).

28. Sibilia,J. *et al.* Structural analysis of human antibodies to proteinase 3 from patients with Wegener granulomatosis. *J. Immunol.* **159**, 712-719 (1997).

29. Stegeman,C.A., Tervaert,J.W., de Jong,P.E. & Kallenberg,C.G. Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N. Engl. J. Med.* **335**, 16-20 (1996).

30. Stegeman,C.A. *et al.* Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann. Intern. Med.* **120**, 12-17 (1994).

31. Voswinkel,J. *et al.* Single cell analysis of B lymphocytes from Wegener's granulomatosis: B cell receptors display affinity maturation within the granulomatous lesions. *Clin. Exp. Immunol.* **154**, 339-345 (2008).

32. Voswinkel,J. *et al.* A novel system to test for specificity of B cell receptors from tissue of Wegener's granulomatosis patients. *Clin. Exp. Rheumatol.* **26**, S90-S96 (2008).

33. Voswinkel,J. *et al.* B lymphocyte maturation in Wegener's granulomatosis: a comparative analysis of VH genes from endonasal lesions. *Ann. Rheum. Dis.* **65**, 859-864 (2006).

34. Watts,R.A., Lane,S. & Scott,D.G. What is known about the epidemiology of the vasculitides? *Best. Pract. Res. Clin. Rheumatol.* **19**, 191-207 (2005).

35. Xiao,H. *et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J. Clin. Invest* **110**, 955-963 (2002).