Bayerische Julius-Maximilian Universität Würzburg

Molecular and functional analyses of human synovial B-lymphocytes in Rheumatoid Arthritis

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
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Würzburg, 2000

Eingereicht am: 05. Juni 2000

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Tag des Promotionskolloquiums: 13. Oktober 2000

Doktorurkunde ausgehändigt am:

To Avó Zé, for showing me that rheumatoid arthritis is a hard to bear disease, but one has to keep on fighting against it.

Die Natur hat sich so viel Freiheit vorbehalten, daß wir mit Wissen und Wissenschaft ihr nicht durchgängig beikommen oder sie in die Enge treiben können.

J.W. Goethe, "Maximen und Reflektionen"

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1. INTRODUCTION

„Rheumatoid Arthritis:
(…) inflammatory affection of the joints
not unlike rheumatism…
but differing materially from it. (…)“
Sir Alfred Garrod, 1859

Rheumatoid arthritis is a systemic inflammatory disorder frequently involving first the smaller joints –such as the fingers- and progressively extending to the larger joints, resulting in characteristic deformities (Fig 1). Since it implies the anatomical change of the connective tissue, rheumatoid arthritis is assigned to the collagenous autoimmune diseases.

Figure 1- *Knee joint presenting the typical damage by rheumatoid arthritis with enlargement of the synovial membrane and destruction of cartilage and bone. The insert shows the rheumatoid arthritis synovial membrane obtained after synovectomy. (Both photos are courtesy of Dr A. König)*

1.1. Aims of the present study

1.1.1. Characterisation of the antigen-driven B-lymphocyte maturation and recirculation in rheumatoid arthritis

Although IgV genes in rheumatoid B-lymphocytes have been intensively analysed [26, 28, 54-57] many questions concerning the antigen-driven B-lymphocyte maturation and recirculation remain unanswered. It would be interesting to know whether B-lymphocyte maturation in rheumatoid tissue presents any differences to B-lymphocyte maturation in secondary lymphatic organs. Moreover it would be interesting to know if there exists a restricted number of antigens acting on the lesions of different anatomical sites of the RA patient, and whether B-lymphocytes recirculate between the different joints.

Therefore in the present study IgVH-genes from synovial tissue B-lymphocytes of different anatomical regions (with different times of disease onset) from a RA patient were analysed**.** Furthermore, we included a histopathological analysis and clinical data of local disease activity, which can give a more complete picture of the role B-lymphocytes in the pathogenesis of RA.

1.1.2. Characterisation of arthritogenic antigens

The nature of antigens inducing intrasynovial T- and B-lymphocyte expansion in rheumatoid arthritis (RA) in human beings is enigmatic, and so

far no single joint-specific self-antigen that gives rise to the arthritogenic immune response has been identified.

One possible way to characterize tissue-specific pathogenic antigens (arthritogenic antigens) is to define the reactivity of rheumatoid B-lymphocyte hybridomas with IgV-gene characteristics of antigen activated B-lymphocytes. Using this approach Krenn et al [28] were able more recently to identify a mitochondrial antigen possibly involved in the pathogenesis of RA.

In the present study the synovial B-lymphocyte hybridoma ELB13/3-56 [21] was analyzed for its specific recognition of cartilage antigens. A heptameric peptide of cartilage oligomeric protein (COMP) could be defined as the target structure of somatically mutated IgG produced by the synovial hybridoma ELB13/3-56. Moreover circulating IgGs specific for the COMP-peptide were detectable at statistically significant higher levels in a cohort of RA- compared to osteoarthritis (OA) patients suggesting that this specificity is RA associated and could be used for the development of new diagnostic tools.

1.2. Historical background

Rheumatoid arthritis is the most common and damaging of all types of arthritis. This disease affects about 1% of the world's adult population (onset between 30 and 60 years), involving all racial and ethnic groups [1, 2], being three times more common among women then among men [2, 3].

Surprisingly joint alterations of rheumatoid arthritis cannot be clearly identified in ancient art or literature [2], in contrast to other arthritic diseases

like gout-arthritis (known since the 4th century B.C.). In 1800 A. Landré-Beauvais made the first description of rheumatoid arthritis in his thesis „*Doit-on admettre une nouvelle espèce de goutte sous la dénomination de goutte asthénique primitive?*“. However, only in 1859 the term Rheumatoid Arthritis was coined by the british physician Sir Alfred Garrod. But even then, he used this term to describe other conditions like osteoarthritis. The clear destinction between rheumatoid arthritis -as a form of chronic inflammation- and osteoarthritis –as a form physical wear of the joints- came only in the first decade of the 20th century.

After World War II an intensive investigation began into the immunologic aspects of rheumatoid arthritis. It was triggered by Erik Waaler's [4] discovery in 1940 that adding rheumatoid arthritis serum to sheep red blood cells coated with rabbit antibodies produced marked agglutination. Other studies identified the same behaviour, and in 1948 this unusual serum protein was named rheumatoid factor.

Since the 1960s investigation of rheumatoid arthritis paralleled with the advances in understanding of both humoral and cell-mediated immunity, the use of laboratorial animal models (table 1), and the widespread of molecular biology. Taken together they allowed new aproaches to a more rational treatment of the disease, even though rheumatoid arthritis remains the least understood joint disease.

Name	Rheum Arthritis	COMP* Arthritis	Collagen Arthritis	MRL/lpr Arthritis	Ag-ind. Arthritis	HLA-B27 Arthritis	Adjuvans Arthritis
Arthritogen	???	COMP	Collagen type II	Fas Mutation	mBSA	HLA-B27	Mycobac
Species	Human	Rat	Mouse, Rat, Monkey,	Mouse	Mouse, Rat, Rabbit	Rat	Rat
Gender dependence	3f:1m	yes (f)	no	no	no	???	no
Genetical Predispos.	yes	yes	yes	yes	yes	yes	yes
Symetrical Polyarthritis	yes	yes	yes	yes	no	yes	yes
Extraarticular Events	yes	no	no	yes	no	yes	yes
Incidence	1%	33-100%	60-100%	100%	100%	100%	100%
Chronicity	yes	no	no	yes	yes	yes	no
Involvement Periph. Joints	yes	yes	yes	yes	yes	yes	yes
Involvement Axial joints	yes	yes	no	yes	no	yes	yes
Inflammatory Relapses	yes	no	no	no	induced	no	no
Erosion/ Pannus	yes	yes	yes	yes	yes	yes	yes
Anti-Coll 2 Antibodies	rare	no	many	yes	yes	???	yes
T-cell dependence	yes	yes	yes	yes	yes	yes	yes
Spontaneous appearance	yes	yes	no	yes	no	yes	no

Table 1*- Comparison beteween some animal models of arthritis and human rheumatoid arthritis. Eventhough serving as surrogates to study some of the events in RA, none of them recapitulates accurately all clinical and pathological features of RA. (adapted from [5]; *data obtained from [6])*

1.3. Synovial membrane as the primary inflammation spot

The synovial membrane in healthy individuals pocesses a simple morphology when compared to other tissues from the motion system. It is composed by a synovial cellular layer and a well vasculised connective tissue

made of fibroblasts/ fibrocytes and fat-cells. Therefore, the healthy synovial membrane presents no other immunecompetent cells than the macrophage-like Type-A cells. Thus, it is interesting to see that this tissue is the center of complex immunologically dependent joint diseases.

In rheumatoid arthritis the chronical synovialitis preceeds the joint and tendon destruction, and may be therefore considered a major causative factor for the tissue destruction. The characteristics of this synovialitis depend on disease duration. However its main feature consists of villous thickning of the synovial membrane overlayed by macrophages, neutrophyles, mastocells, granulocytes, and activated synovial fibroblasts[7].

1.4. The role of T-cells and HLA-DRB1 genes in rheumatoid arthritis

The rheumatoid synovial membrane possesses a surface layer of HLA-DR$^+$, CD14$^+$, CD68$^+$ macrophages followed by a layer of fibroblasts [8]. Below these two layers there is a lining of macrophages adjacent to perivascular-located CD4$^+$ T-cells (mainly of memory phenotype: CD29$^+$ and CD45RO$^+$ [9]) and diffuse distributed CD8$^+$ T-cells. Hence, there is an obvious participation of T-cells in the inflammatory process of rheumatoid arthrits. However, the T-cell specificity in the rheumatoid joint is still unknown, mostly due to the difficulties in sampling T-cells from RA patients (e.g.: patients are often on immunomodulatory medication; sampling occurs considerably after inflammation start, thus excluding the analysis of the "initiator" T-cells [10]). Some researchers tried to overcome these problems by analysing the T-cell receptor (TCR) repertoirs of prestimulated RA synovial T-cells. Eventhough

their results pointed to a preferential usage of the gene segments belonging to the Vβ2, Vβ3, Vβ14 and Vβ17 families [11, 12, 13], these results are not totally reliable since *in vitro* stimulation of the cells may lead to artifacts (e.g.: *in vitro* over-expression of a clone that does not play a crucial role in the *in vivo* process).

Another important fact to be considered whyle studying the role of T-cells in RA is the inherited disease susceptibility associated with the class II MHC genes HLA-DRB1*01, HLA-DRB1*04 and HLA-DRB1*10. Recent studies summarized the relative risk to develop RA for individuals carrying these alleles: the relative risk was of 2.3 for the presence of any allele [14], and of 5 for individuals carrying the HLA-DRB1*04 alleles [15]. The main feature of these three HLA-DRB1 alleles is the presence of a common structural motif (shared epitope) in the third hypervariable region of their β1 chains (Tab 2).

Allele	Motif
HLA-DRB1*0401	QKRAA
HLA-DRB1*0404, *0405, *0408, *0101, *0102	QRRAA
HLA-DRB1*1001	RRRAA

Table 2- *Shared epitopes of the HLA-DRB1 alleles [16].*

1.4.1. HLA-DRB1 molecules may shape the T-cell repertoire in RA

To understand the connection between the HLA-DRB1 molecules and rheumatoid arthritis one has to take a closer look into the TCR of αβT-cells. The variable regions of TCR α and β chains are encoded by rearranged

variable, diversity (only in β chains) and joining segments, with additional N diversity sequences [9, 16]. The sequence of the VDJ junction constitutes the CDR3 and is unique to each T-cell clone. After rearranging their TCR genes αβT-cells undergo positive and then negative selection in the thymus. During positive selection αβT-cells are selected for affinity for self class II MHC and self peptides. Those αβT-cells with high affinity for self class II MHC and self peptides are eliminated during negative selection.

Several studies of TCR Vα and Vβ gene-usage showed that HLA-DR genes influence the αβ $CD4^+$ T-cells repertoire both in normal individuals and RA patients [16]. Thus, some authors propose that HLA-DRB1 may control thymic positive and negative selection of αβ $CD4^+$ T-cells. This control may be achieved by presenting self-antigens to autoaggressive T-cells, which will induce an inflammatory response against the integrity of the joints and leading to arthritis [10]. Since the CDR3 of HLA-DBR1*0401 has great homology with proteins from infectious agents (protein gp110 from EBV or DnaJ from *E. coli*) the HLA-DRB1 may also control αβ $CD4^+$ T-cell selection by binding unkown peptides involved in thymic selection [16].

1.4.2. T-cell stimulation leading to joint destruction

Considering the adjacent location of macrophages to T-cells in the synovial membrane and the possible epitope mimicry between infectious agents and HLA-DRB1 molecules, T-cell stimulation leading to joint destruction could follow the pathway:

During viral or bacterial infection antigen presenting cells (APC) like B-cells and macophages stimulate "normal" T-cells against those infectious agents by presenting the foreign antigens and also by the production of pro-inflammatory cytokines by macrophages. This foreign antigen presentation is followed by an immune response against the infectious agents. However, the infectious agents may have epitopes that mimic HLA-DRB1 molecules. Thus, leading to an "aberrant" T-cell stimulation by presentation of such epitopes to auto-reactive T-cells that escaped thymic selection. These auto-reactive T-cells recruite and stimulate auto-reactive B-cells and macrophages. The auto-reactive B-cells produce antibodies directed against joint structures (discussed in detail in chapter 1.5.), and the auto-reactive macrophages have an over secretion of TNF-α and IL-1 that are responsible for bone erosion (TNF-α) and articular damage through production of metalloproteinases, collagenase and degradation of proteoglycans (IL-1) [8]. Moreover, the overproduction of IL-1 and TNF-α results in a cytokine imbalance that supports the differentiation of defective dendritic cells, which present self-antigens to the auto-reactive T-cells, thus contributing for a raised failure in self-tolerance [17]. All these processes lead to joint inflammation and destruction releasing self-antigens that feed the auto-immune process, in a sort of "vicious circle".

1.5. Synovial B-lymphocytes in rheumatoid arthritis

Intrasynovial B-lymphocytes (including B-lymphocytes and plasma cells) are a constant and dominating component (Fig 2A and D) of the

inflammatory infiltrate in rheumatoid arthritis (RA) [7, 18, 19]. Histopathologically, the synovial B-lymphocytes may exhibit a follicular distribution (Fig 2A and B) and may be located in the area of bone cartilage destruction (Fig 2E), and finally there is a correlation between the serum level of rheumatoid-factors (RF) and the follicular organisation of the synovial B-lymphocytes [19].

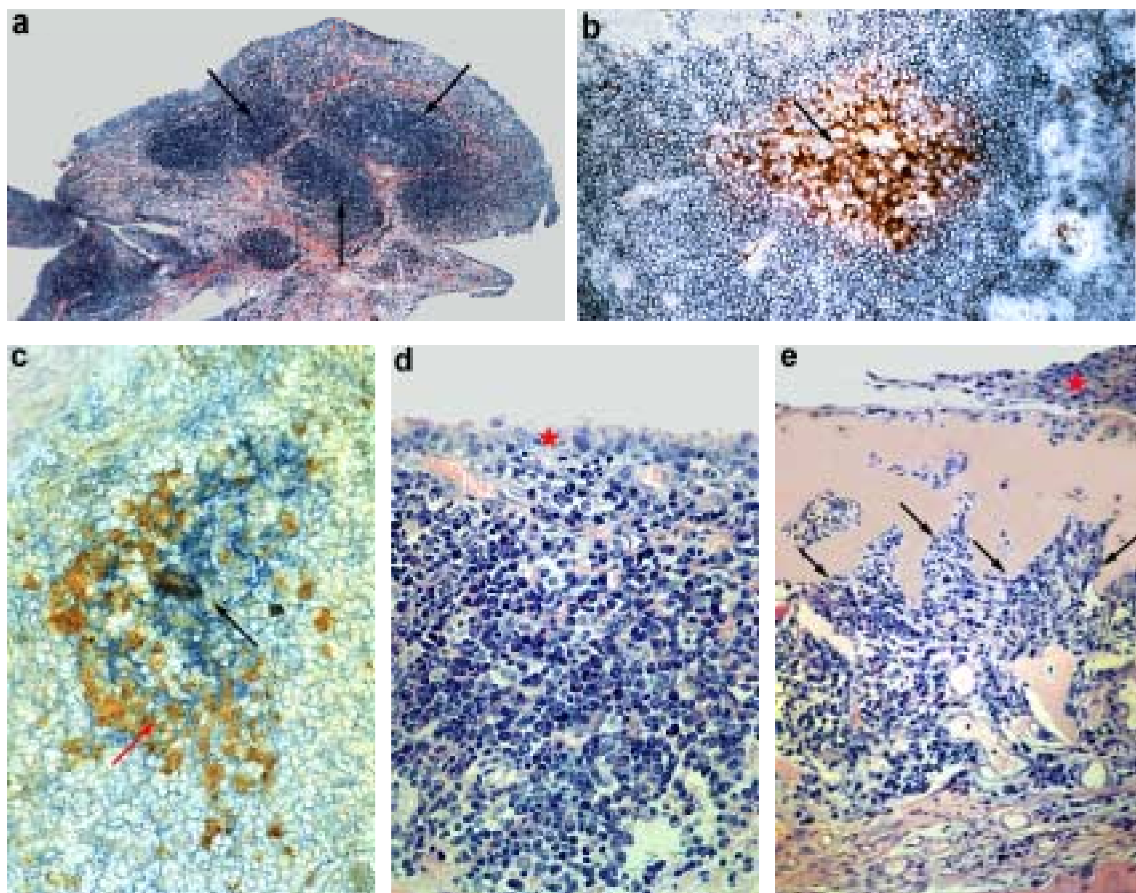

***Figure 2-** Histopathology and histology of rheumatoid synovial tissue. **A**: rheumatoid synovial tissue with villous hypertrophy and lymphofollicular (arrows) inflammatory infiltration (type I, according to [7]); **B**: double staining immunohistochemistry, demonstrating a lymphatic follicle with centrally located FDCs (Ki-M4+; brown area, arrows), and with peripherally located CD20+ B-lymphocytes (intensely blue area); **C**: double staining*

*immunohistochemistry, demonstrating a zonal organization of a germinal center with compartment of proliferating cells (brown area; red arrow), and an area of FDCs (blue area; black arrow) with a reduced number of proliferating cells; **D**: plasma cells rich synovitis (star is located in the enlarged synovial intima); **E**: plasma cell-rich inflammatory infiltrate in the area of bone/cartilage destruction (arrows). Magnification orig: A 50x; B and E 100x, C and D 250x.*

These findings indicate a pathogenic role of synovial B-lymphocytes in RA. Analysis of the pathogenetic potential of synovial B-lymphocytes in RA leads to two important questions: 1. Does a local antigen-activated B-lymphocyte maturation occur in the synovial tissue? 2. Against which antigens are these B-lymphocytes directed?

1.5.1 The organisation of synovial B-lymphocytes

The inflammatory infiltrate of chronic RA synovialitis is basically constituted by the functional elements of a secondary lymphatic tissue, including T- and B-lymphocytes, macrophages as well as antigen-presenting cells (APC). Therefore, the question arises as to whether these cellular elements represent an appropriate level of morphological organisation to permit an antigen-activated B-lymphocyte maturation. In specialized structures of the lymphatic organs (Fig 3A), the so called germinal centers (GC) of the secondary follicle, antigen-activated B-lymphocyte affinity maturation takes place [20, 21]: unactivated B-lymphocytes (naive B-lymphocytes) and a subpopulation of CD4-positive T-lymphocytes [22] come

into close contact with the network-like organized cells of the GC, the follicular dendritic reticular cells (FDC). These cells, together with accessory receptors (CD40; MHC II; ICAM-1), present the antigen to the B-lymphocytes. This cellular interaction leads to cell proliferation and immunoglobulin gene hypermutation (IgVH and IgVL genes), which modifies the affinity repertoire of the B-lymphocytes (affinity maturation) [20, 21]. Finally, the B-lymphocytes with lower affinity for the presented antigen are destroyed (ex: via FasL), and the ones with higher affinity are expanded (affinity selection).

The so-called post-GC B-lymphocytes represent antigen-activated B-lymphocytes with a specific set of B-lymphocyte-receptors with either low or highly diversified VH-genes [20, 23]. This process occurs in the secondary lymphatic organ B-lymphocytes, where high-affinity (post-GC B-lymphocytes) are produced under the control of FDCs, which represent the antigen-induced and T-cell-dependent immune response [23, 24]

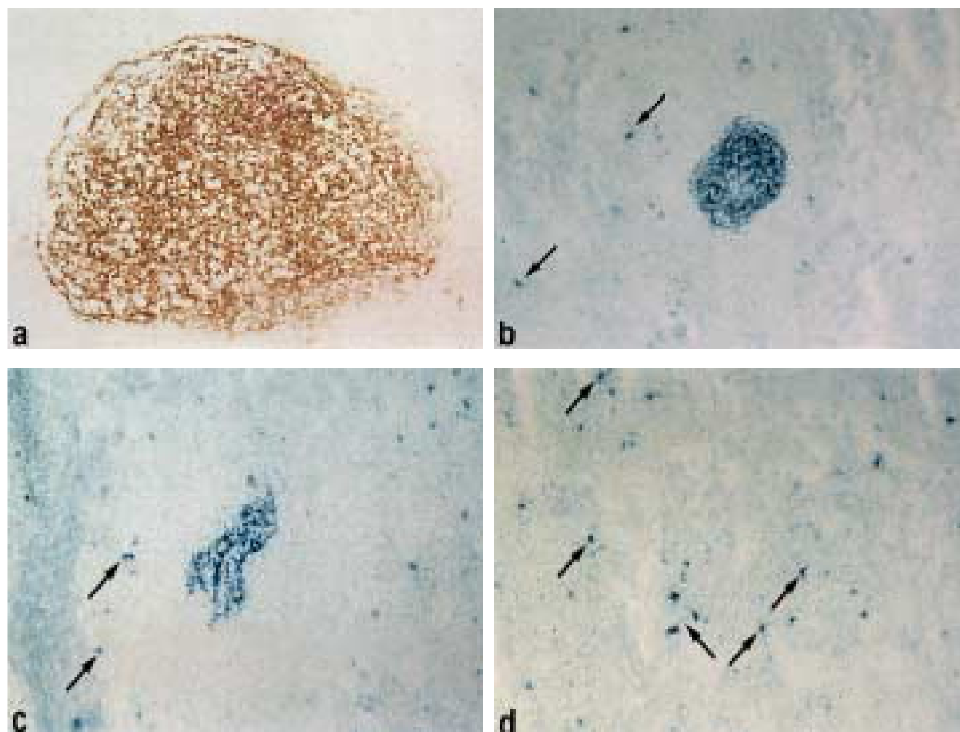

Figure 3- *Patterns of FDC distribution in: (**A**) normal lymphatic tissue (tonsil) and rheumatoid synovial tissue exhibiting a germinal center pattern; (**B**) a germinal center-like pattern and (**C**) a diffuse distribution of single FDCs (**D**). Arrows point at single FDCs. Magnification orig.: 100x.*

The RA synovial tissue often reveals a highly organized cellular composition reminiscent of that seen in the lymphatic tissue of secondary lymphoid organs [10, 19, 25]. The pattern of FDC distribution may be immunohistochemically classified [10]: 1. GC pattern (Fig 3B); 2. GC-like pattern (also called ”dysmorphic follicle”) (Fig 3C); and 3. Diffuse distribution pattern of single FDCs (Fig 4D).

Rheumatoid synovial tissue with immunohistochemically detectable true germinal centers (secondary follicles) occurs only in a small percentage of cases. In a morphometric analysis it was shown that the size of these germinal centers is similar to the size of germinal centers found in the spleen and different from the ones in tonsils and lymphnodes (H. Harms and P. Fretter, unpublished results).

Much more common are the small follicle-like FDC formations (the GC-like pattern). These are small, irregularly shaped FDC formations without zonal organisation. Moreover, there exists a diffuse distribution pattern of single FDCs, which are partially localized perivascularly, but also close to the synovial lining cells.

Finally, in about 30% of all cases, it is not possible to detect any FDCs in the synovial tissue. Through simultaneous detection of the FDC-specific antigens KIM4 and FasL, it has been shown that FasL is expressed in FDC

formations in synovial tissue, indicating that FDC formations in the RA synovial tissue may be involved in negative selection [10].

The existence of secondary follicles and follicle-like structures in the synovial tissue indicates that an intrasynovial antigen-driven maturation of B-lymphocytes may take place. Using a single cell isolation method, recent studies [26, 27] have proven experimentally that a local maturation of non-mutated B-lymphocytes into highly mutated plasma cells occurs in the synovial tissue of RA. B-cells and plasma cells did not carry identical rearrangements, but were clonally related, indicating that B-lymphocytes underwent a terminal differentiation in the synovial tissue (Fig 4).

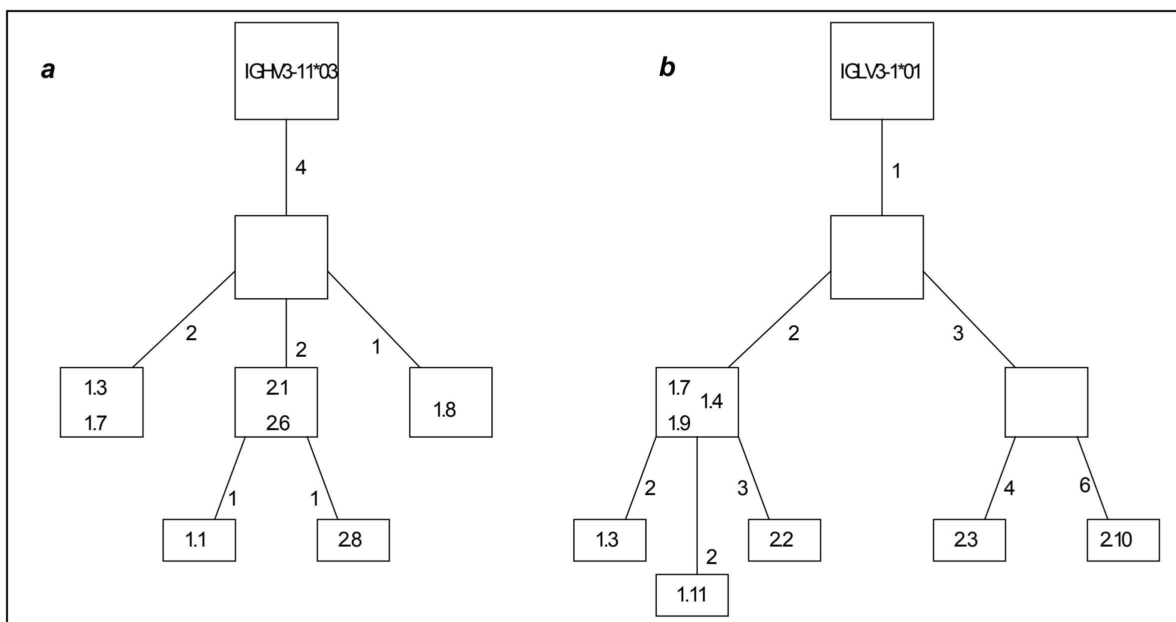

Figure 4- *Step-by-step accumulation of somatic mutations. Diversification on Vgenes is shown for a (A) heavy chain (IgVH3-11*03) and a (B) light chain (IgVL3-1*01) rearrangement. Numbered boxes indicate isolated sequences; empty boxes indicate hypothetical intermediates. Numbers besides lines refer*

to the number of nucleotide exchanges that distinguish one sequence from another. Adapted from [27].

The findings obtained from synovial tissue are well in line with the findings obtained from rheumatoid synovial B-cell hybridomas. Depending on the specificity of the B-lymphocyte hybridomas, unmutated and highly mutated IgVH genes could be detected. In one of these hybridomas of known specificity (to a mitochondrial antibody [28], produced in our laboratory) the values of the R/S ratios in the CDR were higher than 2.9, which indicates that these B-lymphocytes belong to the pool of post-GC B-lymphocytes [21, 29, 30].

The analysis of B-lymphocyte clones from blood, synovial fluid, and synovial tissue with RF-specificity (immunoglobulins with specificity for the Fc portion of immunoglobulins) shows low mutated IgVL-gene sections, so that RF-producing B-lymphocytes belong both to the classes of post-GC and naive B-lymphocytes. Thus, the formation of RF is to be seen in part as antigen-dependent and in part as antigen-independent.

It may be concluded that B-cells and plasma cells exhibit a complex morphological organization in RA synovitis, which can be compared to the microenvironment of peripheral lymphatic organs, where antigen-induced affinity maturation takes place. The characterisation of synovial B-lymphocyte specificities could, therefore, help to identify antigens which are involved in local B-lymphocyte expansion and immunopathogenesis of RA.

1.5.2. The repertoire of synovial B-lymphocyte specificities

The specificity spectrum of B-lymphocytes described in the literature is wide and ranges from serum antibodies to experimentally produced B-lymphocyte clones from peripheral blood, bone marrow, synovial fluid, and synovial tissue [21, 31, 32, 33, 34, 35]. The following specificities have been defined: specificity from hybridomas; RF [34]; ANCA's and ANA's [36]; collagen type II [37, 38]; HLA-DR [39]; thyreoglobulin, tetanusoxoid, DNA, and actin [40]; human [18] and bacterial HSP [18, 38], filaggrin [41]. These specificities can be divided into two specificity groups: antibodies with specificity against "self antigens", and antibodies with specificity against "non-self antigens".

1.5.2.1. Synovial B-lymphocytes with specificity for "self antigens"

Antibodies which exhibit specificity for a self antigen are not necessarily pathogenic. These antibodies may not be the cause, but may also be the consequence of a disease, since liberated antigens may induce an immune response during tissue destruction (e.g.: antibodies against myocardial components after myocardial infarct). Most of these autoantibodies possess a physiological function in binding antigens which are harmful to the organism and lead to antigen clearance. The existence of autoantibodies in immune disease is, consequently, not necessarily connected to a pathogenic role of the antibody. However, many of these antibodies have a high prevalence for defined diseases, and pocess a

diagnostic importance (anti-M2 antibody in PBC; anti-dsDNA antibody in SLE), even though the pathogenic function remains unclear.

An autoantibody may be defined as pathogenic if it fulfills the Witebsky-Koch criteria [42]: 1. Disease induction with transfer of the autoantibody; 2. Autoantibody isolation from the disease-specific lesion; and 3. Disease induction through immunisation with the autoantibody (idiotypic induction). These criteria are only fulfilled by a few autoantibodies occuring in myasthemia gravis, pemphigus vulgaris, autoimmune thrombocytopenia, and Morbus Basedow, which are classed as "classical autoimmune diseases".
For the antibodies which will be described in this work, these criteria have not been totally fulfilled, mainly due to the lack of a suitable animal model for RA.

1.5.2.2. Polyreactive antibodies from synovial B-lymphocytes

Antibodies with specificity for several different antigens are described as polyreactive antibodies. Polyreactive antibodies often have autoantibody specificity and are mainly produced by CD5-positive B-lymphocytes. As “natural antibodies” they fulfill an important function in the primary immunological reaction against bacteria and viruses through their "polyreactivity". Interestingly enough, in the synovial membrane the number of $CD5^+$ B-lymphocytes is quite elevated [43]. Does an elevated number of B-lymphocytes, producing polyreactive antibodies, have a pathogenic role? Since $CD5^+$ B-lymphocytes are regarded as naive B-lymphocytes with germline configuration of their IgVH genes, the participation of $CD5^+$ B-

lymphocytes in synovialitis could reflect the genomic dependence of this disease (association of RA to certain groups).

$CD5^+$ synovial B-lymphocytes could also contribute to antigen clearance. Antigens which are liberated during joint destruction could be recognized by $CD5^+$ synovial B-lymphocytes, focusing the inflammatory reactions to the joint.

1.5.2.3 Monoreactive antibodies from synovial B-lymphocytes

Rheumatoid-factors: These were the first autoantibodies described in RA [4]. They show a specificity for the constant region (Fc region) of an immunoglobulin (IgG). The affinity of the synovial and serum RF diverges among the different IgG-subclasses (IgG1 - IgG4). For example, RF from synovial B-lymphocytes show high affinity for IgG3 [19]. An immunohistochemical analysis showed that about 70% of the synovial IgM-producing B-lymphocytes, 50% of the IgG-producing B-lymphocytes, and 20% of the IgA-producing B-lymphocytes are RF-producing cells [44]. Thus RF are mainly produced by the IgM and IgG subtypes. RF are regarded as pathogenic mainly due to clinical observations, because the RF serum level particularly among older patients is directly correlated to the disease's activity. RF show capacity for "self-organisation" by forming rheuma factor complexes, which have the capacity to activate complement and are detected at the site of tissue destruction [45].

RF are also detected in the blood of healthy individuals. These RF are mainly polyreactive and may not be pathogenic, due to their low affinity. This

implies that pathogenic RF must have a different origin and structure. Recently Sutton et al [46] proposed some answers to the above question based on the crystal structure of a monoclonal IgM RF bound to its target IgG. They suggest that pathogenic RF: 1) bind antigen and IgG Fc simultaneously using the conventional antigen binding site and an adjacent site; 2) are a consequence of antibody responses to infectious antigens or other autoantigens; 3) result from V-gene somatic mutation that does not affect the "classical" antigen binding sites. Furthermore they suggest that the RF receptor on B-cells binds both antigen and IgG Fc in a ternary complex and together with T-cell help lead to a high level RF production.

Monospecific and high affinity RF may contribute to the pathogenesis of RA [47], but that does not explain why the disease is primarily restricted to the joints. Though this joint restriction could be explained by the fact that RF localize in the synovia as complement-activating ternary complexes, with the RF affinity for IgG Fcenhanced by the presence of antigens [46].

Antibodies against type II collagen: They could be regarded as "organ-specific" antibodies, hence collagen II is exclusively expressed in joint cartilage. Collagen-induced arthritis in mice demonstrates the relevance of B-lymphocytes for the pathogenesis of cartilage destruction. At first, destruction may be induced by the passive transfer of collagen type II-specific antibodies [48]; additionally, this disease may be induced through the immunisation with triple helical collagen, which is exclusively recognized by B-lymphocytes, but not by T-cells [49]. Only B-lymphocytes have the exclusive function to recognize conformation-dependent antigens.

In RA, it is necessary to establish a subspecification into pathogenic and non-pathogenic type II collagen antibodies, since these antibodies are found both in the blood of RA patients and healthy individuals. A pathogenic function of antibodies specific for type II collagen could be due to a difference in the fine-specificity between healthy controls and afflicted patients.

Antibodies against mitochondrial antigens: The analysis of a synovial B-lymphocyte clone produced by electrofusion showed –according to indirect immunofluorescence technique- a mitochondrial pattern in the stomach mucosa, which was then confirmed by immune electron microscopy [28]. Western blot analysis of a mitochondrial preparation showed specificity for a mitochondrial antigen, which is related to the M2 antigen. The M2 antigen is an organ-unspecific ATPase-associated antigen, and antibodies specific for M2 are 97% associated with primary biliar cirrhosis (PBC).

The molecular analysis of the IgVH/IgVL genes demonstrated R/S ratios of 2, indicating that the B-lymphocyte had undergone an antigen-induced germinal center reaction. It may be hyphothesized that mitochondrial antigens, which are locally liberated (probably by joint destruction), induce a response to the mitochondrial antigen, perpetuating a local inflammatory reaction. Autoantibodies with specificity for intracytoplasmatic antigens could therefore perpetuate the inflammatory process, but this does not explain the organ specificity of the disease, since these antigens are expressed ubiquitously.

1.5.2.4. Synovial B-lymphocytes specific for "non-self antigens"

In recent years, evidence has accumulated that different forms of bacterial heat shock proteins (HSP) may play a pathogenic role in RA, by exhibiting an antigenic mimicry of "non-self" and "self" components [50]. The remarkable conservation of amino acid sequences between bacterial and human HSP [51] might explain why immune responses initially directed against HSPs from an infectious agent would lead to autoimmune diseases. HSPs, therefore, provide a link between immunity to bacterial infections and autoimmune diseases. T- as well as B-lymphocytes from rheumatoid synovial fluid and tissue were shown to be specific for bacterial HSPs and human [18, 38, 52]. Since HSPs are expressed in the synovial tissue, a humoral HSP60 response initially directed against an infectious agent could cause synovialitis by cross-reactivity. Here again, the question is why the cross-reactivity is restricted to synovial tissue, since HSPs show a ubiquitous expression.

A recent study, performed by Kowal et al. [53] with SLE patients, analysed monovalent antigen-binding fragments reacting with pneumococcal polysaccharide, DNA, or both, and observed that some of these fragments reacted with both self and foreign antigen. They concluded that at the molecular level a molecular mimicry might exist between bacterial and self-antigens. Perhaps a similar approach for RA patients could help to clarify the relation between this disease and bacterial HSPs or other bacterial antigens.

2. MATERIALS AND METHODS

2.1. Patients

2.1.1. Patient, disease activity, and tissue samples (IgVH gene analysis)

Tissue samples (1 from the right another from the left tendon of m. peronei longi and 1 from the cubita synovial tissue) from a 48-years-old female patient with confirmed seropositive RA [58] were obtained at synovectomy and snap-frozen. The patient was receiving antirheumatic medication (gold, methotrexate, and sulphasalazine). In the present investigation, the degree of local disease activity was scored according to the method of Fuchs et al and Krenn et al [10, 59], on the basis of: (i) warmth, (ii) effusion, and (iii) swelling. The patient was seropositive for rheumatoid factors (RF).

2.1.2. Patients, tissue and blood samples, and histopathological analysis (ELB 13/3-56 study)

Synovial tissues were obtained at synovectomy and arthroplasty from patients (n=5) with confirmed seropositive RA [58] and from patients (n=5) with definite idiopathic osteoarthrosis [60]. Human hyaline cartilage of normal joints (knee) were obtained from autopsy of patients (n=3) without joint diseases. The serum samples were obtained from 22 RA patients with confirmed seropositive RA, 24 patients with definite idiopathic OA, and 20 age

matched healthy controls. All RA patients were receiving antirheumatic medication (gold, methotrexate and sulphasalazine).

All patients gave their informed consent and the ethics review committee of the University of Würzburg approved the study.

Rheumatoid arthritis: Histopathological evaluation exhibited in all cases (n=4) the characteristic morphology of long standing rheumatoid arthritis with villous hypertrophy of synovial tissue with marked edema, enlargement of synovial lining and a variable degree of inflammatory infiltration ranging from 3 to 5 according to the inflammatory score of Krenn et al [21, 61].

Osteoarthrosis: Histopathological evaluation exhibited a moderate villous hypertrophy with moderate enlargement of synovial lining, moderate fibrosis, moderate incorporation of cartilage fragments and a variable degree of inflammatory infiltration of 1 and 3 according to the inflammatory score of Krenn et al [61].

2.2. Immunohistochemistry:

For immunohistochemical staining 7-µm cryosections and 5-µm deparaffinated tissue sections (mounted on poly-L-lysine-coated slides) were used. Immediately before staining, the cryosections were treated with acetone for 10 min, air-dried at room temperature (10-20 min), and the following immunohistochemical procedures were performed as described in [21], briefly:

(a) The indirect immunoperoxidase technique involved incubation of the slides with the following primary monoclonal antibodies (mAbs): CD3, CD22 (To15), CD68=Ki-M8, Ki-M4, kappa, lambda, IgM, IgA, IgG and Ki-67 (DIANOVA, Hamburg, Germany) used in dilutions of 1:300, 1:200, 1:5000, 1:500, 1:10, 1:10, 1:200, 1:200, 1:300 and 1:500. Negative controls were always obtained on parallel slides by replacing the primary mAb with PBS or neutral mAb (pancytokeratin).

(b) The alkaline phosphatase anti-alkaline phosphatase technique was performed in the same manner using a rabbit anti-mouse bridging serum. Fast blue salt was used, giving a bright, intense blue staining (CD22, Ki-M4). No counterstaining was performed.

(c) The combined immunostaining was performed by combining (a) the indirect immunoperoxidase technique (Ki-67) with (b) the alkaline phosphatase anti-alkaline phosphatase technique (Ki-M4). No counterstaining was performed. In all cases, control staining was performed and single stainings were compared with double stainings in order to ascertain that the pattern of immunohistochemical reaction remained unaltered.

2.3. Immunohistochemistry of human cartilage with ELB 13/3-56

For immunohistochemical staining 7-µm cryosections of human cartilage (knee) were used. Indirect immunoperoxidase technique was carried out as previously described [61] and involved incubation of the slides with 100µl of ELB 13/3-56 culture supernatant in a 1:50 dilution. Negative controls

were always obtained on parallel slides by replacing the primary mAb with PBS.

2.4. Histopathological score of inflammatory infiltration:

A portion of tissue (approximately 50%) was fixed in formalin and paraffin-embedded (Giemsa, haematoxylin/eosin staining) for use in diagnosis and scoring of the degree of the inflammatory infiltration, which in this study was performed according to Krenn et al [3] on a semiquantitative 1-5 scale. Very low inflammatory infiltration was indicated by 1 on the scale: the synovial intima is slightly enlarged (two to three cell layers thick); the degree of lymphocytic infiltration is very low, showing a diffuse pattern; and the subsynovial region exhibits chronic tissue granulation with slight fibrosis. Low inflammatory infiltration was indicated by 2 on the scale: the synovial intima is slightly enlarged (two to three cell layers thick), and the degree of inflammatory infiltration is low, with a diffuse perivascular lymphocytic and plasma cell infiltration; the subsynovial region shows chronic tissue granulation with moderate fibrosis. Moderate inflammatory infiltration was indicated by 3 on the scale: the synovial intima is moderately enlarged (three to five cell layers thick), and the degree of lymphocytic infiltration is moderate, with small follicle-like aggregates near small blood vessels; there is moderate cellularity of the subsynovial region, which exhibits slight fibrosis. Strong inflammatory infiltration was indicated by 4 on the scale: the synovial intima is extensively enlarged (five to ten cell layers), and lymphocytes exhibit a dense follicle-like pattern; the "interfollicular" area exhibits very high cellularity

without fibrosis. Very strong inflammatory infiltration was indicated by 5 on the scale: the synovial intima is extensively enlarged, and the distribution of lymphocytes exhibits a dense follicle-like pattern with formation of germinal centers; granulomas and hemigranulomas can be seen in the subsynovial region. In each histopathological analysis, ten fields were examined, and the most prominent finding in a given field determined the score.

2.5. PCR amplifications

2.5.1. RNA-isolation from cryosections with RNeasy-Kit and cDNA synthesis

RNA was prepared from 50x 5µm tissue sections using a Rneasy-Kit (Qiagen, Germany) according to the suppliers instructions. The only material used was tissue that exhibited macroscopic signs of inflammation, taken from at least three different regions of the resected synovial membrane.

5µg RNA were mixed with 1µl Oligo-dT_{15} (1µg/µl, MWG Biotech) and 2µl random primer (40µM) in a 10µl volume. This was followed by RNA denaturation for 10 min at 65°C. The sample was cooled down on ice, and then added to a 17 µl Mastermix containing: 5.2µl DEPC-H_2O, 5µl 5x Reverse-Transcriptase-Buffer, 2.5µl dNTPs (each 10mM), 2.5µl DTT (250mM), 0.8µl RNAse-inhibitor (400U, MBI Fermentas) and 1µl M-MLV Reverse Transcriptase (200U, Gibco BRL, USA). CDNA synthesis was carried for 70min at 37°C, followed by enzyme inactivation at 95°C for 5 min.

2.5.2. PCR amplification of cDNA

The amplification of the VH genes from cDNA was carried out in a 25µl volume containing 1.75mM $MgCl_2$, 0.4pM primer, 1U Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany), and 200µM of each dNTP. The cycle profile for amplification consists of DNA denaturation at 95 °C for 2 min, followed by 45 cycles of 94 °C for 30 sec, primer annealing at 65 °C for 30 sec for VH3 and VH4 primers (60 °C for VH1, VH2 and VH5), and extension at 72 °C for 80 sec. Primer sequences were described previously [62], they are located from codon 17 to 24 (according to V-Base [63] sequence-comparison. In brief, the following primers, given in the 5`-3`direction, were used:

V_H1	5´ CCT CAG TGA AGT YTC CTG CAA GGC	3´
V_H2	5´ GTC CTG CGC TGG TGA AAS CCA CAC A	3´
V_H3	5´ GGG GTC CCT GAG ACT CTC CTG TGC AG	3´
V_H4	5´ GAC CCT GTC CCT CAC CTG CRC TGT C	3´
V_H5	5´ AAA AAG CCC GGG GAG TCT CTG ARG A	3´
V_H6	5´ ACC TGT GCC ATC TCC GGG GAC AGT G	3´
J_H1-5	5´ GGT GAC CAG GGT BCC YTG GCC CCA G	3´
J_H6	5´ GGT GAC CGT GGT CCC TTG CCC CCA G	3´

2.5.3. DNA extraction and amplification of IgV_H genes by nested PCR

DNA extraction and amplification of IgV_H genes was performed according to the method of Kim et al. [27] with minor modifications. Briefly: DNA was prepared by incubating 10x5µm tissue sections at 56°C overnight

with 60µl Higushi-Buffer containing 5µl proteinase K (Boehringer, Mannheim, Germany), which was inactivated by heating for 10min at 95°C. To improve the specificity of the PCR amplification, semi-nested PCR reactions were carried out as follows. In the first step, amplification with Taq polymerase was performed with V_H 5´primers and external J_H region specific 3´primers [27]. In the second round, aliquots were specifically amplified for the heavy-chain genes using the same 5´V_H region primers but internal J_H region primers (semi-nested PCR). In brief, the following primers, given in the 5`-3`direction, were used:

V_H1 5' CCA TGG ACT GGA CCT GGA 3'

V_H2 5' ATG GAC ATA CTT TGT TCC AC 3'

V_H3 5' CCA TGG AGT TTG GGC TGA GC 3'

V_H4 5' ATG AAA CAC CTG TGG TTC TT 3'

V_H5 5' ATG GGG TCA ACC GCC ATC CT 3'

V_H6 5' ATG TCT GTC TCC TTC CTG AT 3'

$J_H$external 5' CTC ACC TGA GGA GAC GGT GAC C 3'

$J_H$internal 5‘ TGA (AG)GA GAC GGT GAC C(AG)(GT) GT(GCT) CC 3‘

The final concentrations of the reagents were 0.1 mM $MgCl_2$, 200 µM of each dNTP, 10 pM of each primer and 2 U Taq DNA polymerase. The cycle program consisted of a denaturation step at 95°C for 5 minutes followed by 5 cycles at 95°C for 40 seconds, 65°C for 40 seconds and 72°C for 1 minute and 50 seconds; 5 cycles at 95°C for 40 seconds, 60°C for 40 seconds and 72°C for 1 minute and 50 seconds; 25 cycles at 95°C for 40 seconds, 55°C for

40 seconds and 72°C for 1 minute and 50 seconds. The cycles were followed by a final 10 min incubation at 72°C.

2.5.4. PCR-amplification with ELB 13/3-56 specific primers

Genomic DNA was prepared as described above. The amplification of the V_H genes was carried out in a 25 µl volume containing 1.75 mM $MgCl_2$, 0.4 pM primer, 1 U Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany), and 200µM of each dNTP. The cycle profile for amplification consisted of DNA denaturation at 94°C for 90 sec followed by 40 cycles of 94°C for 90 sec, primer annealing at 62°C for 60 sec, and extension at 72°C for 90 sec. Following primers specific for the VH-genes of ELB13/3-56 [21] were used (given in the 5'-3' direction):

ELB-For 5‘ GAC CCT GTC CCT CAC CTG C(AG)C TGT C 3‘;

ELB-Rev 5‘ GTA GAC AAA ATA ACT CCC CGA ATT AAA TG 3‘

2.6. Ligation of the PCR products to the plasmid vectors

Aliquots of the final PCR products were separated by electrophoresis using a 2% low melting agarose gel (Roth, Karlsruhe, Germany) , and DNA bands in the range of 350 bp were purified from the agarose gel using High-Pure DNA gel extraction kit (Boehringer, Mannheim, Germany). Ligation of PCR products was performed with pCR-Script Amp SK(+) cloning kit (Stratagene, Heidelberg, Germany) or pGEM-T Easy Cloning kit (Promega, Mannheim, Germany) according to the suppliers instructions.

2.7. Preparation of competent DH5α *E. coli*

About 10 colonies of DH5a E. coli were added to 500ml LB-medium and incubated at 37°C until $OD_{550} \approx 0.5$. It followed a 30min incubation at 4°C and than a centrifugation step of 2000xg for 15min at 4°C. The pellet was resuspended in 20ml chilled buffer RF1 (100mM RbCl, 50mM $MnCl_2$, 30mM KAc, 10mM $CaCl_2$, 15% (w/v) Glycerin, pH 5,8, sterile filtred) and icubated for 20min at 4°C. The cells were than pelleted by a 2000xg centrifugation for 9min at 4°C and resuspended in 20ml RF2 buffer (10mM RbCl, 10mM MOPS, 75mM $CaCl_2$, 15 % (w/v) Glycerin, pH 6,8, sterile filtred). After 15min incubation at 4°C the cell suspension was aliquoted (200µl) into sterile 1ml caps and snap-frozen in a dry ice/ ethanol bath.

2.8. Transformation of competent bacteria

Competent bacteria were thawed on ice. 100µl bacteria were mixed with 4µl of the ligation product into a sterile 15ml tube and incubated on ice for 30min. After a heat-shock for 45sec at 42°C they were cooled down on ice for 2min and then mixed with 900µl SOC-medium (980µl SOB-medium, 20µl 10%(w/v) Glucose). After 60min incubation at 37°C with gentle shaking (250 rpm) 100 and 200µl of the transformation product were plated on ampicillin (50µg/ml) LB-Agar-plates previously coated with 40µl IPTG/X-Gal/Dimethylformamid. The plates were incubated overnight at 37°C.

2.9. Mini-plasmid isolation

Each white colony was added to 3ml LB-medium containing 3µl ampicilin (50µg/ml) and icubated overnight at 37°C with gentle shaking. The mini-plasmid isolation was carried out with 1.5ml of the overnight culture using a Jet-Prep Kit (Genomed, Bad Oeynhausen, Germany) according to the suppliers instructions.

2.10. Restriction analyses

0.5µg plasmid was digested for 60min at 37°C with 1µl of each restriction enzyme (MBI Fermentas, Ucraine), 1µl 10x reaction buffer and 3µl. Afterwards 4µl sample were separated by electrophoresis in a 2% agarose-gel (Roth, Karlsruhe, Germany). Plasmids carrying an insert were than sequenced.

2.11. IgVH gene sequence analyses

Positive clones were sequenced using the DyeDeoxy Termination Cycle Sequencing Kit (Applied BioSystems Inc., Weiterstadt, Germany), and analyzed with an automated DNA sequencer ABIPrism373. Both strands were sequenced using T3 and T7 primers:

T_3 5' ATT TAA CCC TCA CTA AAG GG 3'

T_7 5' GTA ATA CGA CTC ACT ATA GGG C 3'

The sequences were analyzed using DNAman for Windows software (Lynon Software, Canada), Genebank and v-Base databases [63].

2.12. Antibody purification

The human monoclonal rheumatoid synovial B-cell hybridoma ELB13/3-56 was produced at the Institute for Pathology from the University of Würzburg as previously described [21].

250ml of culture supernatant of the hybridoma ELB 13/3-56 were loaded, according to the suppliers instructions, in a 1ml HiTrap rProtein A column (Pharmacia Biotech). The column was then washed with five column volumes of 20mM Sodium-Phosphate buffer pH 7. The bound antibody was eluted with 3 column volumes of 100mM Sodium-Citrate buffer pH 4.0, and 1ml aliquots were collected in tubes previously containing 200µl of 1M TrisCl pH 8.0. The concentration of the purified antibody was determined by the Bradford protein quantification method using the Rotiquant system (Roth).

2.13. Immunoblotting

50µl of cartilage extracts were diluted in sample buffer (containing 50mM TrisCl pH 6.8, 2% SDS) under reduced (2% v/v β-mercaptoethanol) and unreduced conditions, and applied to 12% polyacrylamide gels, and run according to Laemmli [64]. The samples were transferred to a nitrocellulose membrane by electrophoresis in a semi-dry transfer cell (Bio-Rad), according

to Towbin et al [65]. The antigens were revealed using the ELB 13/3-56 antibody and by enhanced chemiluminescence (Pierce).

2.14. Immunopercipitation

50µg of the purified ELB 13/3-56 antibody was incubated overnight at 4°C with 200µg cartilage extract, 10µl Prot A agarose (BioRad, Germany), 400µl IP-buffer (containing 1%TritonX-100, 150mM NaCl and 50mM TrisCl ph 8.0) and 500µl distilled water. After centrifugation for 30sec at 13000 rpm the supernatant was discarded and the pellet was washed 3 times with IP-washing buffer (containing 0.1%TritonX-100, 150mM NaCl and 50mM TrisCl ph 8.0). Then the pellet was resuspended in 50µl reducing sample-buffer (containing 50mM TrisCl pH 6.8; 2% SDS and 2% β-mercaptoethanol) and incubated for 5 min at 958C. After centrifugating for 1min at 13000 rpm the supernatant was transferred into a fresh tube.

2.15. Silver-staining of the SDS-PAGE gel

The sample obtained by immunoprecipitation was run in a 12% polyacrylamide gel according to Laemmli [64]. The gel was then silver stained after a modified method of Shevchenko et al [66], in order to reduce the background staining and to enhance the staining of smaller bands. Briefly: the gel was fixed for 60min in a 25% methanol / 25% ethanol / 5% $C_2H_3NaO_2$ solution. This was followed by a first washing step with 25% methanol / 25% ethanol for 30min, and a second one with distilled water also for 30min. Then

it was sensitised for 5min with 0.02% $Na_2S_2O_3$ and washed twice for 15min with distilled water. The staining proceeded with a 30min incubation with 0.1% $AgNO_3$ / 0.02% formaldehyde. After a final washing step with distilled water for 15min the bands were revealed with 2% Na_2CO_3 / 0.04% formaldehyde. The reaction was stopped with distilled water. The whole procedure was carried out at room temperature with gentle shaking.

The specific band was cut off and stored at 4°C in distilled water until sequencing.

2.16. Protein Identification

The protein was identified by nanoelectrospray mass spectrometry [67] after tryptic digestion as previously described [68]. The peptide mixture was dried down and dissolved for mass spectrometric work in 2.5 µl methanol/water/formic acid (50:49:1, v/v/v).

The MS measurements were performed with a Q-Tof (Micromass, Manchester, UK) equipped with a nanoflow Z spray ion source. To identify the protein we used the sequence tag program which combines partial manual spectrum interpretation of about three amino acids (sequence tag) with the residual mass *N*- terminal and *C*-terminal of the interpreted region and the peptide mass to search in a non-redundant translated nucleotide database. [69].

2.17. COMP Purification

COMP purification was carried at the Department for Connective Tissue Biology of the University of Lund (Sweden) according to their established protocol [70]. Briefly: 30g Human cartilage was dissected with a bisturi on a cold dissection bench. The dissected cartilage was then homogenized at full speed (two cycles of 1min) in 10 volumes extraction buffer I (0.15M NaCl, 0.1M ε-aminocaproic acid, 5mM benzamidine/HCl, 5mM Tris, 10mM N-ethyl-malemid, pH 7.4). The homogeneized cartilage was than extracted for 1 hour at 4C with gentle shaking.

After a 20min centrifugation at 20000 rpm and 4°C the pellet was resuspended in 12 volumes extraction buffer II (0.15M NaCl, 10mM EDTA 0.1M ε-aminocaproic acid, 5mM benzamidine/HCl, 5mM Tris, 10mM N-ethyl-malemid, pH 7.4), and extracted over night at 4°C with gentle shaking. The extract was then centrifuged for 20min at 20000 rpm and 4°C. The pellet was extracted over night at 4°C with 12 volumes guanidine extraction buffer (4M Guanidine-HCl, 20mM Tris-HCl, pH 8.0).

To check out in which extraction step COMP was present, a 4%-16% gradient SDS gel was run after Laemmli [64] with 0.5mg supernatant-protein per well, and then immunoblotted using a rabbit anti-bovine COMP policlonal antibody (courtesy of Prof. D. Heinegård) as previously described. As expected, COMP was mainly present in the supernatant of the 2nd extraction. Therefore, this supernatant was diluted 1:1 in H_2O, and loaded over night in a 35ml DEAE-sepharose column (Pharmacia Biotech) previously equilibrated

with 3 column volumes of elution buffer A (5mM Tris-HCl, 1mM EDTA, 0.075 M NaCl, ph 7.4).

The proteins were eluted over night using a linear growing salt gradient obtained by mixture -with a gradient mixer (Pharmacia Biotech)- of 250ml elution buffer A and 250ml elution buffer B (5mM Tris-HCl, 1mM EDTA, 0.5 M NaCl, ph 7.4). The samples were collected in 6ml fractions. To assure that the salt gradient grew linearly the conductance of each second fraction was measured. The absorbance of each fraction was measured at 280nm and 50µl of each fraction with the highest absorbance (fractions 42-50) was run on a 4%-16% gradient SDS gel under reduced and unreduced conditions to check out for the presence of purified COMP.

2.18. Phage display

The Ph.D.-7 phage display peptide library kit (New England Biolabs, USA) was used for mapping the epitope(s) recognized by ELB 13/3-56. Two rounds of biopanning were carried out according to the suppliers instructions. Briefly: petri dishes were incubated overnight at 48C with 100mg/ml of ELB 13/3-56, and after being blocked with the blocking solution (0,1M $NaHCO_3$, 5mg/ml BSA and 0,02% NaN_3) they were incubated with $2x10^{11}$ phage from the original library. The bound phages were then eluted with 0,2M glycine-HCl (pH 2,2), and amplified in the *E. coli* strain ER2537. With the amplified phage a new round of biopanning was carried out. At the end of this second round the bound phage were transfected into the ER2537 *E. coli*, and the

heptameric peptid determined through automated cycle sequencing (ABIPrism 373, Applied BioSystems, USA) using following primer:

-96 5' CCC TCA TAG TTA GCG TAA CG 3'

The consensus peptid sequence was compared with the whole COMP sequence, to determine the location of a possible epitope within the protein.

2.19. Epitope-ELISA:

The 96-well plates were incubated over-night with the potential COMP epitope "KDPRNVG" (100µg/ml in PBS). After being blocked with RPMI-medium, the wells were coated with 200µg/ml serum samples of RA (n=22) and OA (n=24) patients and healthy controls (n=20) diluted in PBS. Afterwards the peroxidase-conjugated rabbit anti-human IgG secondary antibody (Dako, Denmark) was added. It followed a detection step using the substrate solution (4 OPD-Tabs (Dako, Denmark), 14ml Citrate-Buffer pH2 and 25µl H_2O_2). After a 10 minutes incubation, the reaction was stopped with 50µl 3M H_2SO_4. The plates were read using a microplate reader (Model 550, Bio-Rad, Germany) set at 490nm. The same procedure was repeated using purified ELB 13/3-56 in plates coated either with the potential COMP epitope or with the heptameric control peptide PVGNDRK (the control peptide was chosen based on its marked hydrophylic and hydrophobic differences to the potential COMP epitope (fig. 5)).

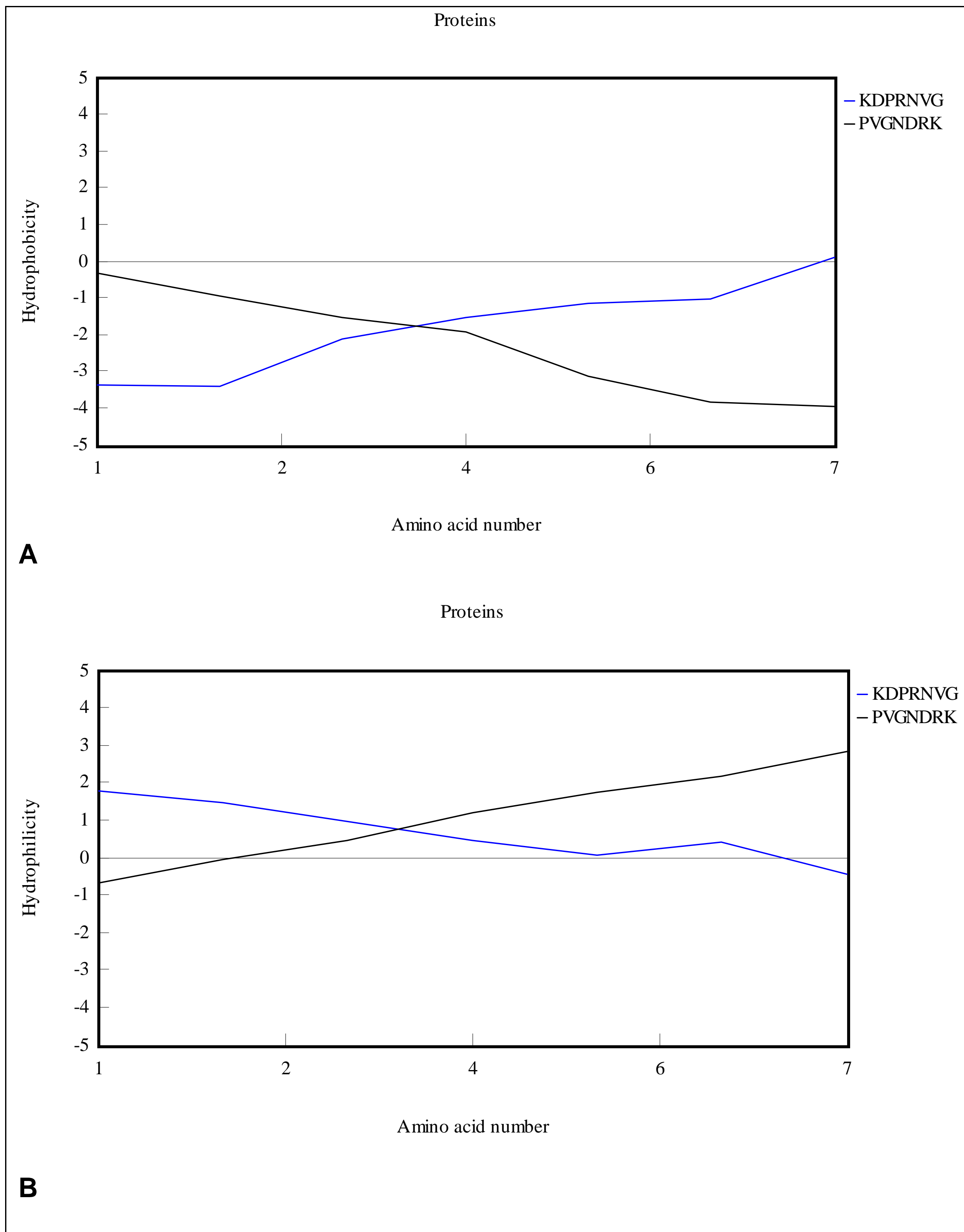

Figure 5- *Graphic overview of the different hydrophobic (A) and hydrophilic (B) properties of the potential COMP epitope (KDPRNVG) and the control peptide (PVGNDRK).*

2.20. Photographic Material

For the photographic documentation of the immunohistological stainings it was used a ®AFGA RSX 50 film. The photographs were shot in a ®Zeiss Axiophot.

3. RESULTS

3.1. Characterisation of the antigen-driven B-lymphocyte maturation and recirculation in rheumatoid arthritis

3.1.1. Local disease activity and duration of local disease (table 3)

The female patient, who suffered from a confirmed seropositive RA with involvement of tendon sheaths, exhibited severe signs of local disease activity (swelling +++, tenderness +++), with tenosynovitis of the right distal peroneus longus tendon (a), left distal peroneus longus tendon (b) and synovialis of the right cubita (c). The duration of local disease was 5 months for (a), 2 months for (b) and 2 weeks for (c).

Localisation	Disease Duration	Local Disease Activity		Inflammation	Pattern	Presence	ΣR/ΣS	
	(weeks)	Swelling	Tenderness	Score	of Infiltration	of FDCs	CDR	FR
a) right peroneal tendon	20	(+++)	(+++)	5	follicular	(+)	7.50	1.48
b) left peroneal tendon	8	(+++)	(+++)	5	follicular	(+)	3.65	1.70
c) right cubita	2	(+++)	(+++)	2	diffuse	(-)	3.00	1.39

Table 3- *Comparison between local disease activity and molecular data*

3.1.2. Histopathology and immunohistochemistry of synovial tissue

A heterogenous inflammatory infiltrate could be observed in the different localisations. In both localisations with longer disease duration (a) (right peroneal tendon, 5 months) and (b) (left peroneal tendon, 2 months) a very intense inflammatory infiltrate with Ki-M4 positive FDC-containing germinal centers (fig 6a, 6b and insert) could be observed (inflammatory score 5), whereas in localisation (c) (right cubita, 2 weeks), a low, diffuse non-follicular infiltration with marked oedema (inflammatory score 2) could be detected (fig 6c).

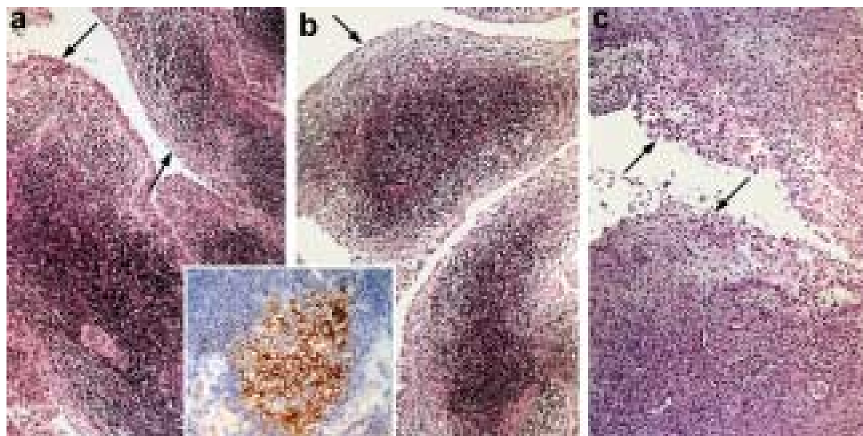

Figure 6- *Histopathology (HE) and immunohistochemistry (double staining: indirect immunoperoxidase and alcaline phosphatase in insert of 1a and 1b) of rheumatoid synovial tissue from three different anatomical localisations of the RA patient.* ***A****- right peroneal tendon sheath;* ***B****- left peroneal tendon sheath with inserted figure showing Ki-M4 positive FDCs' network (brown) surrounded by CD20^{+} B-lymphocytes (blue) representing a germinal center;* ***C****- synovial membrane from the right cubita. Arrows point at enlarged synovial intima (original magnification 350x).*

The later synovialitis showed histopathologically a more acute inflammatory reaction, while in (a) and (b) the morphological pattern of a typical chronic tendosynovitis was present. Immunohistochemically (a) and (b) exhibited a dense follicular-like infiltration with Ki-M4-positive FDC and peripherically located CD20-positive B-lympocytes representing germinal centers (insert in fig 6a and 6b). In (c) only a very low and diffuse non-follicular distribution of lymphocytes without Ki-M4-positive FDC could be recognized.

3.1.3. Comparison of the mutated VH segments with the germline genes

3.1.3.1. Presence of pseudogenes (Table 4)

From the 55 analyzed clones, 7 expressed non-functional rearrangements (pseudogenes) with stop codons, and 48 were found to express functional genes. The existence of pseudogenes has been largely described in IgV genes amplified from genomic DNA of healthy [71, 72] and diseased [73, 74] individuals. Furthermore the presence of pseudogenes in diseased individuals is largely related to specific mutations on the RYGW motifs [73]. Based on these findings, it is not surprising for us to have found pseudogenes in the genomic DNA amplificates (k194/81; k194/120; k194/126). However we also obtained pseudogenes in the cDNA amplificates (k194/30; k194/33; k194/130; k194/135), which have not been yet described in literature. These pseudogenes could be the product of a PCR artifact (maybe due to an elevated number of cycles) that introduced incorrectly

STOP codons on the IgV-gene sequence. But since the sequences were read in both directions (5'-3' and 3'-5'), and both readings yielded the same confirmatory results, we cannot consider the pseudogenes in the cDNA amplificates as PCR artifacts. The existence of such pseudogenes could be explained by the findings of Drapkin et al [75] that DNA repair enzymes are part of the RNA polymerase II transcription initiation process. Hence, there could be a defective DNA repair mechanism that in our special case could lead to the introduction of STOP codons in the RNA molecule.

Localisation	Clone	VH-Family	Germline	Homology %
l.p.t[a].				
	K194/1[#]	1	IgHV1-18*01	79.4
	K194/3	1	IgHV1-69*01	73.6
	K194/4	1	IgHV1-3*01	96.6
	K194/5	1	IgHV1-69*01	66.9
	K194/6	1	IgHV1-18*01	88.2
	K194/8	2	IgHV2-70*11	98.1
	K194/9	2	IgHV2-70*01	92.0
	K194/23	4	IgHV4-30-1/4-31*02	100.0
	K194/24	3	IgHV3-53*02	95.1
	K194/25	4	IgHV4-59*01	91.0
	K194/26	4	IgHV4-30-4*06	89.0
	K194/27	5	IgHV5-51*01	96.1
	K194/28	5	IgHV5-51*01	85.0
	K194/30[##]	5	IgHV5-51*01	P
	K194/31	5	IgHV5-51*01	93.8
	K194/33	5	IgHV5-51*01	P
	K194/42	5	IgHV5-51*01	98.0
	K194/81	2	IgHV2-70*01	P
cubita				
	K194/57[##]	1	IgHV1-18*01//IgHV1-46*01	ND
	K194/58	1	IgHV1-46*01	66.5
	K194/62	1	IgHV1-18*01	91.6
	K194/67[##]	1	IgHV1-18*01//IgHV1-2*02	ND
	K194/72	1	IgHV1-69*01	95.3
	K194/76	1	IgHV1-18*01	97.9
	K194/77	1	IgHV1-8*01	94.5
	K194/79	1	IgHV1-18*01	75.7
	K194/82	1	IgHV1-46*01	80.3
	K194/88	1	IgHV1-2*02	91.2
	K194/89	1	IgHV1-3*01	86.0
	K194/92	1	IgHV1-8*01	88.7

	K194/95	1	IgHV1-69*01	76.9
	K194/98	1	IgHV1-8*01	96.2
	K194/99[##]	2	IgHV2-70*01	92.0
	K194/100	4	IgHV4-30-1/4-31*02	97.0
	K194/101	4	IgHV4-30-1/4-31*02	97.0
	K194/102	4	IgHV4-30-1/4-31*02	97.0
	K194/103	4	IgHV4-30-1/4-31*02	98.0
	K194/104	6	IgHV6-1*01	88.0
r.p.t.[a)]				
	K194/109[##]	1	IgHV1-8*01//IgHV1-18*01	ND
	K194/111[#]	1	IgHV1-18*01	77.0
	K194/114	1	IgHV1-18*01	79.0
	K194/117	1	IgHV1-18*01	93.0
	K194/118	1	IgHV1-18*01	88.0
	K194/119[##]	1	IgHV1-18*01	96.0
	K194/120	1	IgHV1-18*01	P
	K194/121	2	IgHV2-70*01	97.0
	K194/122	5	IgHV5-51*01	94.0
	K194/123	5	IgHV5-51*01	87.0
	K194/124	5	IgHV5-51*03	92.0
	K194/125	5	IgHV5-51*03	98.0
	K194/126[##]	5	IgHV5-51*01	P
	K194/127	5	IgHV5-51*01	94.0
	K194/130	5	IgHV5-51*01	P
	K194/135	5	IgHV5-51*01	P
	K194/136	5	IgHV5-51*01	98.0

Table 4- *B-cell clones from the different anatomical localisations are shown with closest identified VH germline gene segment and its homology. a) lpt- left peroneal tendon; rpt- right peroneal tendon; #) clone presenting amino acid deletions; ##) mixed molecule; P) Pseudogene; ND) not determined;* ***bold*** *indicates amplification from genomic DNA.*

3.1.3.2. Deletions and Mixed Molecules

Among the 48 clones expressing functional genes, there were 2 presenting amino acid deletions on their CDR2 -clones K194/1 (Fig 7) and K194/111. These detected deletion events can be regarded as the result of somatic hypermutation -and not as a PCR artifact-, since they were found in

the intrinsic somatic hypermutation hotspots [76-79], and also involved triplets from CDR2. This leaves the transcripts functionally in frame without profoundly altering the backbone structure of the molecule, as defined by Wilson et al [80].

```
                                                                   ___
                     V   S   C   K   A   S   G   Y   T   F   T   S
IgHV1-18*01         GTC TCC TGC AAG GCT TCT GGT TAC ACC TTT ACC AGC
K194/1              --- --- --- --- --- --- --C --- --- --- --- TA-
                                                                 Y

                    _CDR I__________
                     Y   G   I   S   W   V   R   Q   A   P   G   Q
IgHV1-18*01         TAT GGT ATC AGC TGG GTG CGA CAG GCC CCT GGA CAA
K194/1              -T- --- --- TC- --- --- --- --- --- --- --- --C
                     F                                           H

                                            ________________CDR II__
                     G   L   E   W   M   G   W   I   S   A   Y   N
IgHV1-18*01         GGG CTT GAG TGG ATG GGA TGG ATC AGC GCT TAC AAT
K194/1              --- --- --- --- --- --G --- --- XXX XXX AG- -G-
                                                             S   S

                    ______CDR II_________________________________
                     G   N   T   N   Y   A   Q   K   L   Q   G   R
IgHV1-18*01         GGT AAC ACA AAC TAT GCA CAG AAG CTC CAG GGC AGA
K194/1              CAC GGT T-- -C- --- --G -GA --C T-- --C -A- ---
                     H   G   S   T           R   N   F   H   D

                     V   T   M   T   T   D   T   S   T   S   T   A
IgHV1-18*01         GTC ACC ATG ACC ACA GAC ACA TCC ACG AGC ACA GCC
K194/1              C-- CAG C-- --- --- --- --T --- --- --- --- ---
                     L   Q   L

                     Y   M   E   L   R   S   L   R   S   D   D   T
IgHV1-18*01         TAC ATG GAG CTG AGG AGC CTG AGA TCT GAC GAC ACG
K194/1              -T- --- --- --- --- --- --- --- C-- --- --- ---
                     F                               P

                     A   V   Y   Y   C   A   R
IgHV1-18*01         GCC GTG TAT TAC TGT GCG AGA
K194/1              --- --T --C --T --- A-- --G
                                             T
```

Figure 7- *Comparison of IgVH sequence K194/1 obtained from the synovial tissue of a rheumatoid arthritis patient with its closest germline counterpart IgHV1-18*01. The crosses (X) indicate the nucleotid deletions in the patient IgVH-gene. The values of the R/S ratios are 13/2 in CDR and 6/6 in FR.*

Two types of mixed molecules were found. The first type mixed molecules (k194/57; k194/67; k194/109) are composed of rearrangements of two different IgV genes. These two mixed molecules could be considered PCR-artifacts, as the ones described by Bridges et al [81] for amplified Vkappa gene segments in RA synovium. This could be due to the fact that RNA is very unstable and could have fragmented while the samples had not been snap frozen (for hygienical reasons it is not allowed to take the snap-freezing apparatus to the operating theater). However, two facts speak against PCR artifact hypothesis: 1) the B-cells of the RA synovial samples are always used in our laboratory not only for IgV analysis but also for hybridoma production, so that their RNA must be intact to allow a successful cell fusion [21, 28]; 2) the sequences were read in both directions (5'-3' and 3'-5'), and both readings yielded the same confirmatory results of functional mixed molecules.

The second type mixed molecules (k194/126; k194/119; k194/30; k194/99) are composed of a IgV gene rearrangement fragmented by insertions of small random sequences. These insertions are different from the insertions described by Wilson et al [80], since they are not the duplication of parts of the IgV gene. On one hand this could happen due to the insertion of incorrectly amplified fragments, thus resulting in a PCR hybrid artifact. However, the sequences were read in both directions (5'-3' and 3'-5'), and both readings yielded the same confirmatory results. Moreover, the use of nested PCR to amplify the genomic DNA strongly reduces the possibility of amplification and insertion of incorrect fragments [74, 82], thus rendering improbable the hypothesis of a PCR artifact.

Although not considering the deletions and the mixed molecules as PCR artifacts we made the decision not to consider them for further mutational analyses.

3.1.3.3. Local overall R/S ratios raise with time of disease duration (Fig 8)

The 41 in frame functional clones accumulated between 4 and 46 replacements on their amino acid sequence. The R/S ratios in the CDR of all clones from each anatomical region were all higher than 3.

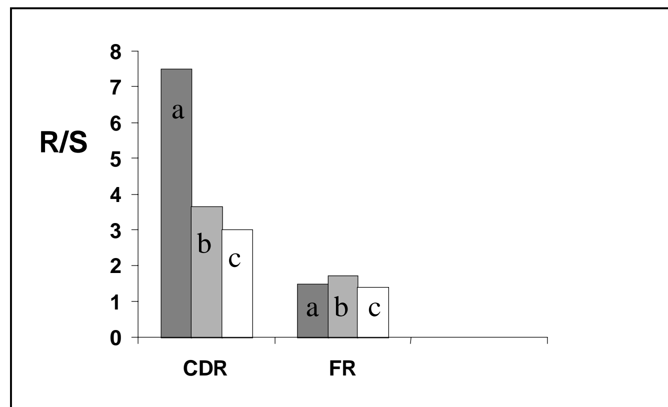

Figure 8- *Total R/S ratios in the FR 1+2+3 and CDR 1+2 of B-cell clones from each different anatomical region of the RA patient: dark gray- right peroneal tendon sheath; light gray- left peroneal tendon sheath; white- cubita.*

There was a direct connection between the R/S values and the time of local disease duration. Locations (a) and (b) with longer disease activity also had higher R/S values in the CDR than location (c) with a later onset.

3.1.3.4. Heterogenity among the CDR3

Even though there was a relatively reduced number of different VH germline gene segments usage, the CDR3s were encoded by D gene segments that differed in both amino acid sequence and length, and all of the six known human JH gene segments were found. As expected for the normal adult Ig repertoire [83], the JH4 and JH6 segments were the most commonly used.

3.1.4. Comparison of the sequences from the same VH family amplified from each location

The comparison was restricted to the VH1 and VH4 families, since they presented the more relevant results.

3.1.4.1. VH1 family

The mutational patterns of immunoglobulin VH1 genes have been studied by Borretzen et al [35] in peripheral blood monoclonal IgM rheumatoid factors of healthy individuals and RA patients. However, this kind of mutational pattern comparison has not been extended to B-cells from RA synovial tissue. As widely known, the primary structure of an antibody, formed by the amino acid sequence determines all its chemical and biological properties. Thus, the amino acid sequences belonging to the VH1-family obtained from the three anatomical regions were primarily compared to the

amino acid sequences of their closest germline counterparts (fig 9a). One result from this comparison is the heterogenity in the CDR3 rearrangements. Moreover, sequences k194/58 and k194/82 are clonally related (confirmed at nucleotide level, data not shown).

```
                          CDR1                          CDR2

IgHV1-2*02   VSCKASGYTFT GYYMH WVRQAPGQGLEWMG WINPNSGGTNYAQKFQG RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR
k194-88      ----------- --fi- -------------- --d----d--------- -----w----------v-spa------i----

IgHV1-3*01   VSCKASGYTFT SYAMH WVRQAPGQRLEWMG WINAGNGNTKYSQKFQG RVTITRDTSASTAYMELSSLRSEDTAVYYCAR
k194-4       ----------- ----- -------------- -------d--------d -----s-------------------------g

IgHV1-8*01   VSCKASGYTFT SYDIN WVRQATGQGLEWMG WMNPNSGNTGYAQKFQG RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR
k194-77      ----------- n---- -----a------l- ----------------- --a---d--k----------------------
k194-89      ----------- ---f- ------------i- ---agn---r-s-n--- --ai--dp-an-v-l-----t-----------
k194-92      ----------- n---- -----a------l- ----------------- --a---d--t---fl--ri-g-d---l-f---
k194-98      ----------- ---f- ------------i- ----------------- --------------------------------

IgHV1-18*01  VSCKASGYTFT SYGIS WVRQAPGQGLEWMG WISAYNGNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR
k194-6       ----------- g-f-h -------------- ----n----h--h---- ----------r----d----------i--gri
k194-62      ----------- n---- -------h------ ---tst-y-h---nfh- -----r----n---------------------
k194-76      ------s---- -f--- -------------- ---------d------- --------------------------------
k194-79      -------d--i nf-f- ------------l- ---n--n---t-pn-r- -ls----p-----fl-----t-n---m----q
k194-117     ----d-----s n-v-- -----h-------- -mn--h--------i-- ---k------------------g---e-----
k194-118     ----------- ghflh ------------v- --npd-------h---- --------------------tpe--s--f-tr
k194-119     ----------- --d-n -----t-------- ----------------- ------------------n-------------

IgHV1-46*01  VSCKASGYTFT SYYMH WVRQAPGQGLEWMG IINPSGGSTSYAQKFQG RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
k194-58      ------t---m n-ci- -l-------p---- v---t----t------- -----k----n--f--vtr-t-a-----f---
k194-82      ------t---m n-ci- -l-------p---- v---t----t--hn--- -----k----n--f--vtr-t-a-----f---

IgHV1-69*01  VSCKASGGTFS SYTIS WVRQAPGQGLEWMG RIIPILGIANYAQKFQG RVTITADKSTSTAYMELSSLRSEDTAVYYCAR
k194-3       --------al- n-s-- -------k------ gt--f-nt----kd-kd ---fst-a-mtvv----tnva---a-i----g
k194-5       -------df-r tnvfa -l-hv----f---- e-r-vfdvirv-er--- ---li--v-a-------hr-sl---s--f-tr
k194-72      ----------- i---- -------------- g----f-t--------- ----st-e--y-v----r--------------
k194-95      -----a-y--t ----- --------ap--v- --t-s-na-dt--r--- --s-s--e--t----s-a--t-d-----f-sk

                  CDR3

k194-88      gggtyrnw        fdp     wgqgtlvt
k194-4       avrqllypdh yyyyymdv     wgqgttvt
k194-77      npaytgd         fdp     wgqgtlvt
k194-89      daegatkaldi             wgqgtlvt
k194-92      gvpsgtnny               wgqgtlvt
k194-98      dygdyrv    yyyygldv     wgqgtlvt
k194-6       praagiryqayryrrprggaryp
k194-62      grresrhivvvta   fdy     wgqgn
k194-76      drapycggdcyst    dy     wgqgtlvt
k194-79      drdsrgsgtwgrlgp         wgqgtlvt
k194-117     dldssswtgery    fdy     wgqgtvv
k194-118     tpgdgwpfyf              wghgtlvs
k194-119     yctstscypg   yyhmdv     wgqgtws
k194-58      tyfdvwsthrdaqdl         wgqgtlvt
k194-82      tyfdvwsthrdalhl         wgqgtlvt
k194-3       aqqlgpyrf    yygvdv     wgqgtlvt
k194-5       tpgdgwpfef              wgqgtpvt
k194-72      gsswysdy   yyyygmdv     wgqgnp
k194-95      ngkagdffe         d     wgqgtlvt
                                                                                           A


IgHV1-18*01  VSCKASGYTFT SYGIS WVRQAPGQGLEWMG WISAYNGNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR
k194-3       -------gals n-s-- -------k------ gtipflnta---kdfkd ---fs--a-mtvv----tnva-e-a-i----g
k194-4       ----------- --amh --------r----- --n-g--d-k-s--f-d ---i-s---a-------s----e--------g
k194-5       -------df-r tnvfa -l-hv----f---- e-rpvfdvirv-erf-- ---lia-v-a-------hr-sle--s--f--t
k194-6       ----------- g-f-h -------------- ----n----h--h---- ----------r----d----------i--gri
k194-58      ------t---m n-c-h -l-------p---- v-nptg-s-t----f-- -----k----n-vf--vtr-t-a-----f---
k194-62      ----------- n---- -------h------ ---tst-y-h---nfh- -----r----n---------------------
k194-72      -------g--s i-t-- -------------- g-ipif-ta-----f-- ---is--e--y-v---------e---------
k194-76      ------s---- -f--- -------------- ---------d------- --------------------------------
k194-77      ----------- n-d-n -----a------l- -mnpns---g----f-- --a--r---k-------s----e---------
k194-79      -------d--i nf-f- ------------l- ---n--n---t-pn-r- -ls----p-----fl-----t-n---m----q
k194-82      ------t---m n-c-h -l-------p---- v-nptg-s-t--hnf-- -----k----n-vf--vtr-t-a-----f---
k194-88      ----------- g-f-h -------------- --dpns-d------f-- -----w---i------vs-pa------i----
k194-89      ----------- --dfn -----t------i- -mn-g----r-s-nf-- --ai-r-p-an-v-l--s--t-e---------
k194-92      ----------- n-d-n -----a------l- -mnpns---g----f-- --a--r-------fl---i-g-----l-f---
k194-95      -----a----- --t-- --------ap--v- r-tpslnaadt--rf-- --sisa-e--t----s-a--t-------f-sk
k194-98      ----------- --dfn -----t------i- -mnpns---g----f-- -----rn--i-------s----e---------
k194-117     ----d-----s n-v-- -----h-------- -mn--h--------i-- ---k------------------g---e-----
k194-118     ----------- ghflh ------------v- --npd-------h---- --------------------tpe--s--f--t
k194-119     ----------- --d-n -----t-------- ----------------- ------------------n-------------
                                                                                           B
```

***Figure 9- A**) Comparison of the translated amino acid sequences of 19 VH1 segments with their closest germline counterparts. **B**) Comparison of the translated amino acid sequences of 19 VH1 segments with germline gene IGHV1-18*01. All sequences have been deposited in Genbank database with following accession numbers: AF209875-209902; AF211924-211932; AF241154-241156 and AF241186-241199.*

Then the 21 amino acid sequences were compared with the widest used germline counterpart IgHV1-18*01 to determine whether a common motif could be discerned (Fig 9b). As postulated, the conservation of the amino acid sequence of all three FR is crucial for the interaction with the antigen [84]. In fact, for FR 1+2 we observed highly conserved regions (Fig 10).

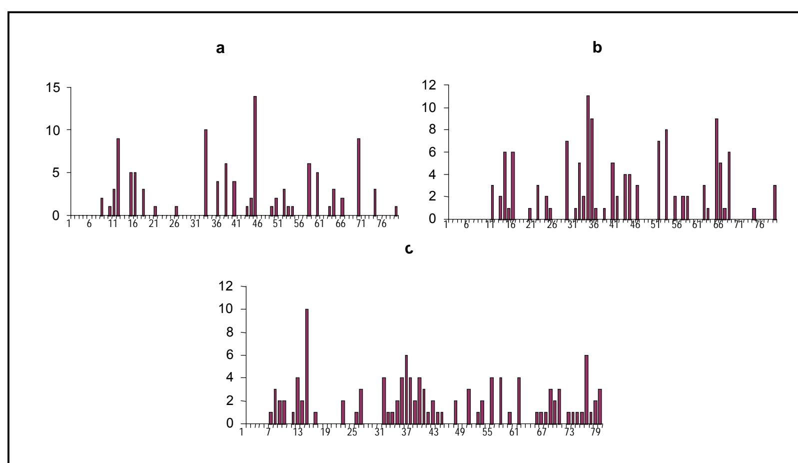

***Figure 10**- Amino acid replacement plot, calculated across translated amino acid sequences shown in Fig 9B. A) Conservative replacement {Y↔ D, E; ↔N D, E; L↔ V, A, F, I; S↔ N, T, Q; D↔ E; K↔ R}; B) Indeterminant replacement {H↔ all amino acids; M↔ all amino acids; G↔ A, V, L, I, F, P; W↔ R, K, S, T, N, Q, D, E; P↔ A, V, I, L,F; K, R↔ N, Q, S, T, Y; C↔ D, E, K, N, Q, R, S, T,*

Y; Y↔ K, R, N, Q, S, T}; C) Non-conservative replacement {R, K↔ E, D; D, Y, E↔ A, I ,L ,P, F; S, T, Q, N↔ A, I, L, V, P, F; R, K↔ A, I, L, P,V, F; C↔ A, I, P, V, L, F; W↔ A, I, P, F, V, L; G↔ C, D, E, R, K, N, Q, S, T, Y}. The type of replacement was defined based on [85-89].

The residues at positions number 6, 10 and 11 from FR1 and the complete FR2 (except positions 22 and 26) showed a high conservation of the amino acid residues. In FR3, we found a high sequence diversity (Fig 9b, 10), even though there was still conservation within residues 48-49, 51-52, 54, 56, 58-60, 62-64, 66-67, 69, 71-75. All the other positions had a total of 30 non-conservative substitutions, and therefore they probably do not play a determining role in the antigen-mediated activation process. As expected, the number of non-conservative substitutions in both CDR1 and CDR2 was extremely elevated. Nevertheless, the 3' end of CDR2, covering residues 41 to 47 presented a total of 25 substitutions, but only 5 were non-conservative (Fig 10). More striking evidence was that residues in position 34 of CDR2 in 11 from 19 replacements resulted in a proline residue. It could even be speculated that position 34 of CDR2 might be an antigen-selected mutational hotspot, since it does not belong to the defined somatic-hypermutation hotspots [75, 76]. In position 45 there are 13 substitutions from Leu to Phe, but 12 of them cannot be considered real substitutions since -as shown in figure 8a- the germline IgHV1-18*01 is the only one to have a Leu in that position instead of the more frequent Phe. Therefore, in this position we only considered Leu-Ile and one Leu-Phe (for k194/62) as real substitutions.

As stated by Chang et al [30], the CDR1 is the IgVH gene region with higher susceptibility to amino acid replacement, and in fact this was the case for all the obtained sequences. The CDR1 had the highest number of non-conservative replacement mutations, which makes it very unlikely to be primarily involved in the antigen-mediated activation.

3.1.4.2. VH4 family

From the 7 amplified sequences of the VH4 family (4 from the cubita and 3 from the left peroneal tendon) 5 had IgHV4-30-1/4-31*02 as their closest germline counterpart and the other two had IgHV4-59*01 and IgHV4-30-4*06 (table 4). When comparing the 5 sequences belonging to IgHV4-30-1/4-31*02 (fig 11a) with each other there was an evidence of two different clonal relations. The first clonal relation was between sequence k194/100 and k194/101 (fig 11b), suggesting that both sequences derived from a single progenitor cell with the rearrangement IgHV4-30-1/4-31*02_IgHD4-17*01_IgHJ5*02. The second clonal relation was between sequences k194/23, k194/102 and k194/103 (fig 11c), suggesting that sequence k194/102 derived from k194/103 which in turn derived from sequence k194/23 that had a progenitor cell with the rearrangement IgHV4-30-1/4-31*02_IgHD2-2*02inv_IgHJ4*01. Furthermore, the little number of mutations of all these 5 sequences when compared to the germline could be taken as an indirect evidence that the germline already encodes a high affinity antibody, as suggested by Williams et al [54].

```
IgHV4-30-1/4-31*02
AGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGCCAGCACCCAGGGAAGGGCCTGGAGTG
k194-100           ------------------------------------------------------------------------------------------------
----
k194-101           -----------------------------------------------------------------------------------t------------
----
k194-23            ------------------------------------------------------------------------------------------------
----
k194-103           ------------------------------------------------------------------------------------------------
----
k194-102           ------------------------------------------------------------------------------------------------
----

IgHV4-30-1/4-31*02
GATTGGGTACATCTATTACAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTCTCCCTG
k194-100           --------------------------a-----t------------------t------------------------g-------------------
----
k194-101           -------------------------------c------------------------tt---t----------------------------------
----
k194-23            ------------------------------------------------------------------------------------------------
----
k194-103           -------------------------------c----------------------------------------------------------------
----
k194-102           -------------------------------c---------------------t------------------------------------------
----

IgHV4-30-1/4-31*02 AAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCG
k194-100           ------------------------------------------------gggactacggtgactacgagagtacttaactggttcgacccctggg
k194-101           ------------------------------------------------gggactacggtgactacgagagtacttaactggttcgacccctggg
k194-23            ------------------------------------------------
agagagggtcctattatagtagtggttaccccgaacgagattgactactggg
k194-103           ------------------c-----------------------------
agagagccgaccgatatagcagctcgtacttgggcctttgactactggg
k194-102           ------------------c-----------------------------
agagagccgaccgatatatcagctcgtacttgggcctttgactactggg

IgHV4-30-1/4-31*02
k194-100           gccaaggaaccctggtcaca
k194-101           gccaaggaaccctggtcaca
k194-23            gccaaggtaccctggtcaca
k194-103           gccaaggtaccctggtcaca
k194-102           gccaaggaaccctggtcaca
```

A

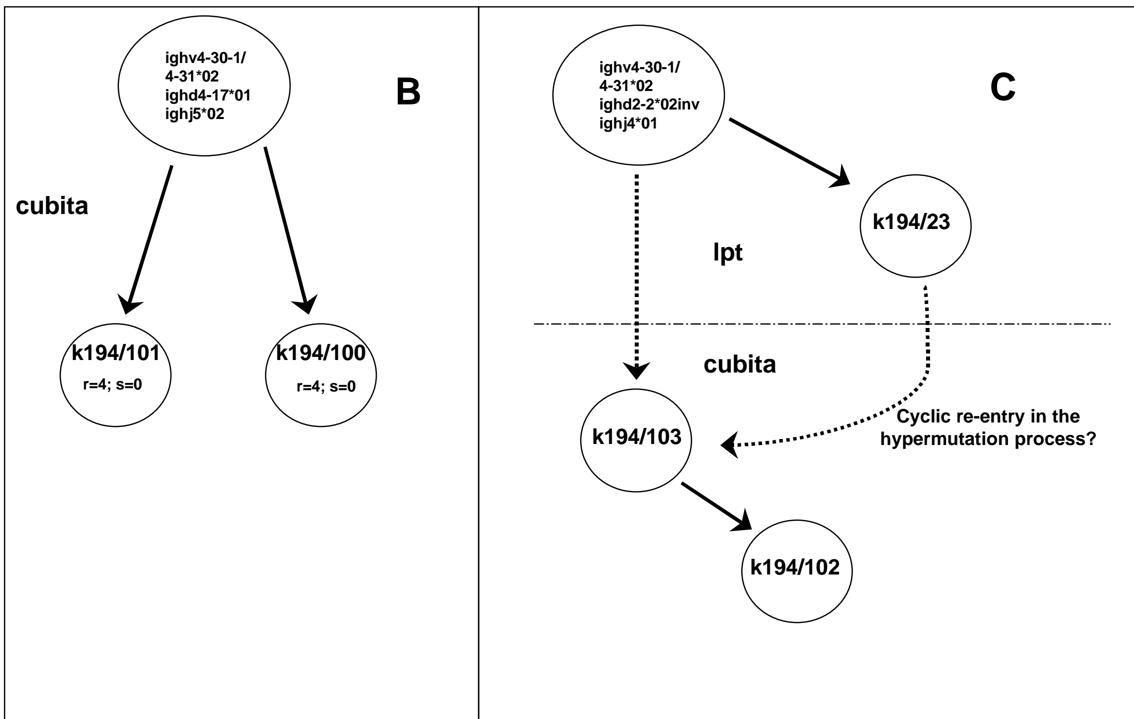

Figure 11- *Analyses of the VH4 family amplificates.* ***A****) Comparison of the 5 nucleotide VH4 sequences with their germline counterpart IgHV4-30-1/4-31*02.* ***B****) Diagram showing the clonal relation between sequences k194/100 and k194/101. Replacement (**r**) and Silent (**s**) mutations on the VH segment when compared to the germline.* ***C****) Diagram showing the clonal relation*

*between sequence k194/23 amplified from the left peroneal tendon (**lpr**) and sequences k194/102 and k194/103 amplified from the cubita. Dashed arrows indicate the possibility of cyclic re-entry of the mature B-cells in the hypermutation process.*

3.2. Characterisation of arthritogenic antigens

3.2.1. ELB13/3-56 is expressed in the synovial membrane of RA patients

ELB13/3-56 is a hybridoma producing an IgG2λ antibody that carries a high number of somatic mutations with high R/S values in the CDR`s indicating that the IgVH-genes have undergone an antigen induced affinity maturation [21]. We designed primers specific for ELB13/3-56 heavy chain genes and performed PCR amplifications with the synovial membrane of RA and osteoarthritis (OA) patients.

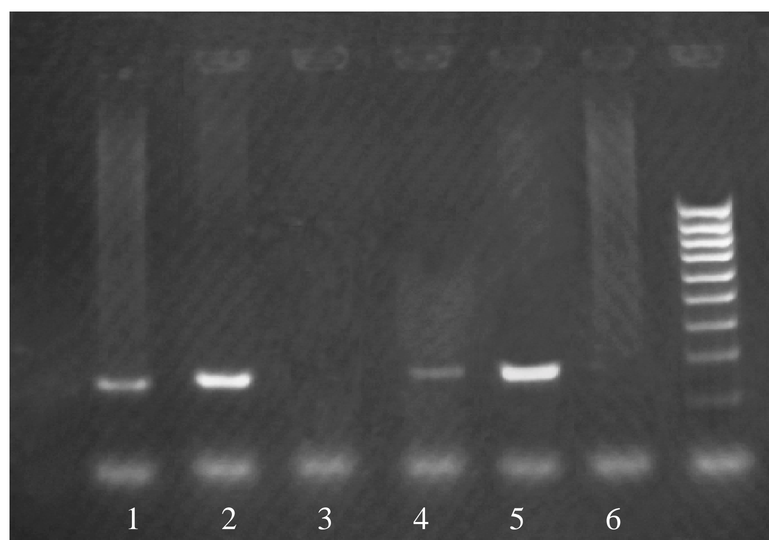

***Figure 12**- PCR amplification of the ELB 13/3-56 specific VH4-gene in RA and OA synovium samples using ELB 13/3-56 specific primers. Lane 1, 2 and 4 RA patients; lane 3 OA patient, lane 5 positive control and lane 6 negative control.*

From the tissue of all 5 RA patients, but from none of the respective OA-derived material we were able to amplify heavy chain genes highly homologous (98%±1%) to the ELB13/3-56-sequences (fig 12). This indicates that B cells closely related to ELB13/3-56 and eventually of the same specificity are frequently present in the inflamed synovial membrane of RA-patients in a disease specific manner.

3.2.2. ELB13/3-56 shows immunereactivity to human hyaline cartilage

Binding of ELB13/3-56 to the human hyaline cartilage could be demonstrated *in vitro* by an immunohistological analysis that revealed a strong and diffuse staining pattern in the interterritorial matrix between the chondrons. In the chondrocytes and the pericellular/ perichondroid matrices no staining could be detected (fig 13A). This staining pattern is in accordance with the immunohistochemical data obtained by DiCesare et al [90] using a rabbit antiserum specific for human cartilage oligomeric matrix protein (COMP). Specificity of the immunohistochemical results obtained with the ELB13/3-56 in our study was proven by the absence of any staining in the respective negative controls (fig 13B).

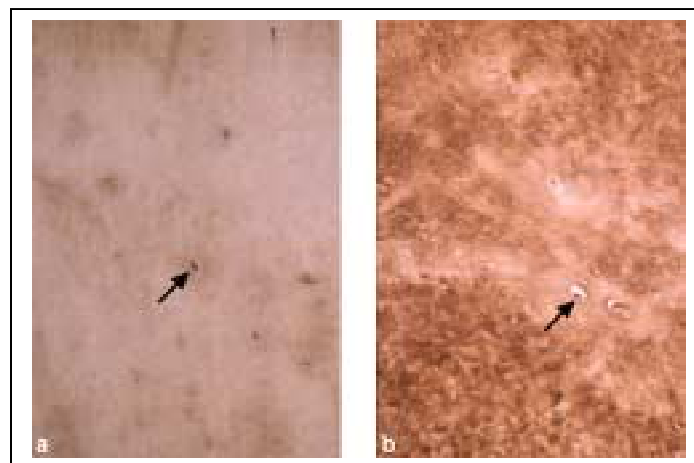

Figure 13- *Immunohistochemical analysis of ELB 13/3-56 on cryosections of human hyalinic cartilage with an intense staining (indirect immunoperoxidase) in the interterritorial matrix between the chondrons (**B**). (**A**) negative control. Arrow points at a chondron (original magnification x150).*

3.2.3. Specific bands in the immunoblot of ELB13/3-56 with cartilage extracts

Immunoblotting experiments of cartilage extracts under reducing and non-reducing conditions revealed the specificity of ELB13/3-56. As shown in figure 13 ELB13/3-56 staining of 3 distinct bands at 60kD, 70kD and 90kD is clearly visible under unreduced conditions. In addition, a smear at 200kD and a band at 500kD that is considerably weaker in the negative control are apparently related to the immunoreactivity of ELB13/3-56 (fig 14).

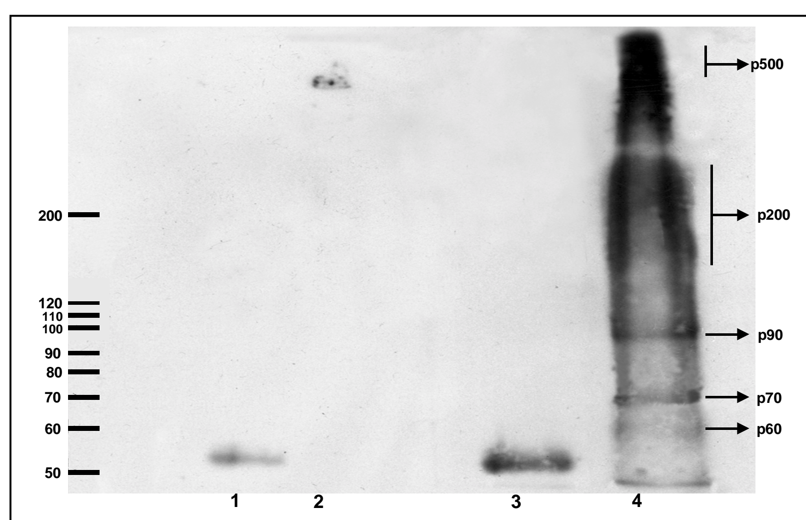

Figure 14*- Western-Blot of ELB 13/3-56 against human cartilage extract under reducing and non-reducing conditions. Under reduced conditions ELB 13/3-56 had no specific reactivity (lane 3); under reducing conditions several specific bands resembling the ones for COMP obtained by Neidhardt et al [91] could be observed (lane 4). Lanes 1 and 2 negative controls (ELB 13/3-56 substituted by PBS) for the reduced and non-reduced fractions respectively.*

The staining pattern of ELB13/3-56 is reminescent of the electrophoretic mobility of the COMP protein under non-reducing conditions as described by Neidhart et al [91]. Whereas the 200kD signal corresponds to the characteristic oligomeric smear obtained for COMP, the 500kD band could represent the pentameric form of this extracellular matrix protein. The bands between 60 and 90kD are within the range of the electrophoretic mobility that

has been described for the α- and, the β-band and a low molecular weight fragment of COMP [91].

Under reducing conditions no specific bands (fig 14) were detectable suggesting the recognition of a conformational epitope that depends on intact di-sulfide bonds by ELB13/3-56.

3.2.4. ELB13/3-56 specifically binds to COMP

Although immunoblotting experiments suggested binding of ELB13/3-56 to COMP, definite experimental proof of antigen specificity was still missing. Therefore cartilage extracts were immunoprecipitated with ELB13/3-56 resulting in a 40kD band that was made visible by silver staining (fig 15A) and further analyzed by sequencing using nanospray tandem mass-spectrometry (table 5). The sequence information from tryptic fragments of the immunoprecipitated protein revealed identity with COMP (fig 15B,C) in consistency with the earlier immunohistochemical and immunoblot results.

A definite proof for the binding of ELB 13/3-56 to COMP was obtained by immunoblotting of the antibody against purified COMP (Fig 15 D).

Mass	Theoretical mass	Residues	Peptide sequence
1025.68	1025.60	629-638	AVAEPGIQLK
1031.56	1031.51	642-651	SSTGPGEQLR
1613.89	1613.77	652-665	NALWHTGDTESQVR
2272.21	2272.08	699-718	FYEGPELVADSNVVLDTTMoxR

Table 5- *Peptide sequences determined by MS/MS. Mox is oxidized methionine,*

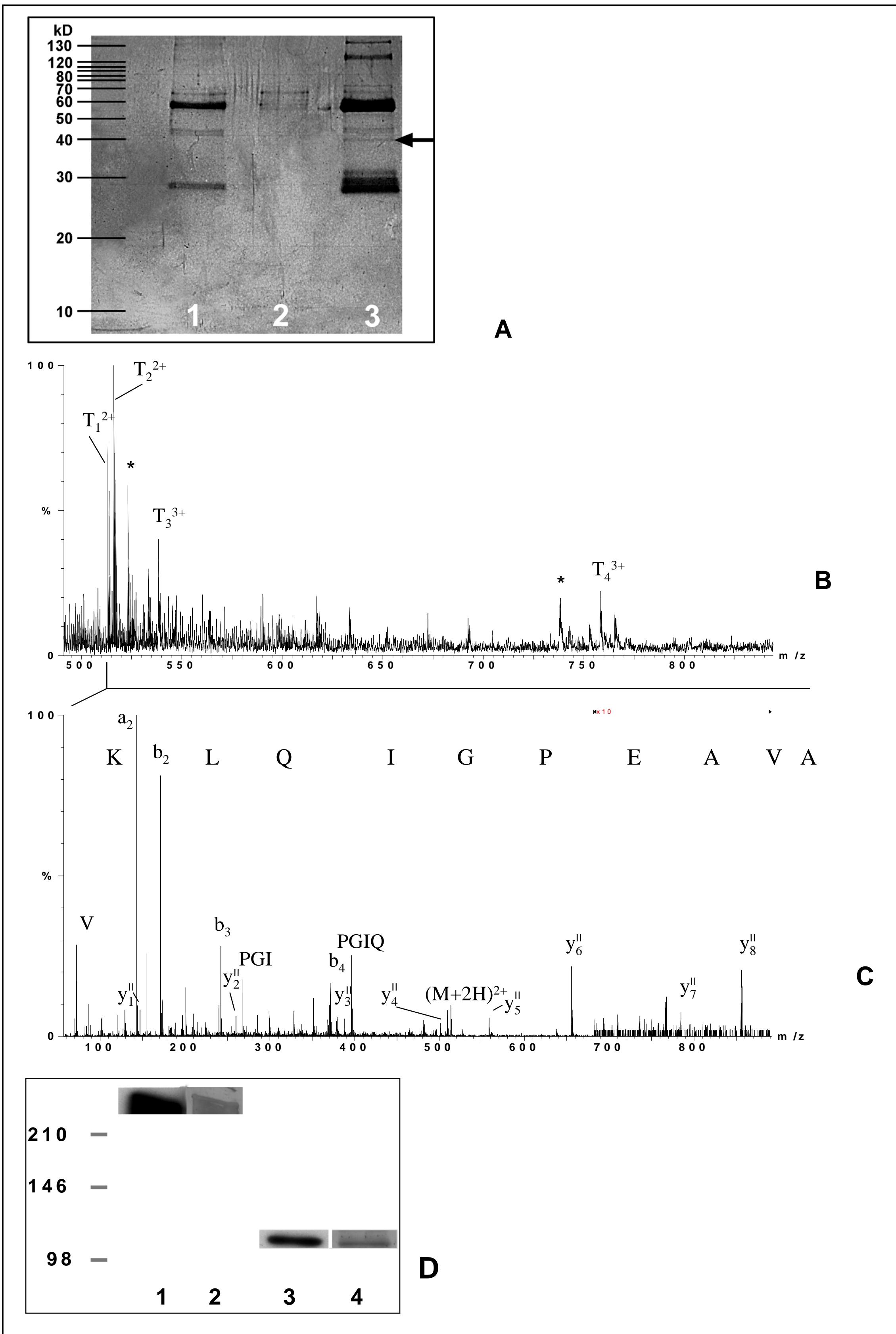

***Figure 15**- (**A**) Immunoprecipitation of ELB 13/3-56 plus human cartilage extract presenting a specific 40kD band in lane 3 (arrow). Lane 1 is the ELB 13/3-56 control and lane 2 is the cartilage extract control.*

*Identification of the silver stained band by nanoelectrospray tandem mass spectrometry. (**B**) Spectrum of the unseparated in-gel tryptic digest. Peaks designated with * belong to trypsin autolysis products, peptide ions designated with T were used for fragmentation in the collision cell to get sequence information (Table 5). (**C**) Tandem mass spectrum of the doubly charged ion T_1^{2+} with a mass to charge ratio (m/z) of 512.84. The peptide sequence was identified using the sequence tag method [68]. In addition, note that the shown sequence reads from the COOH terminus to the NH_2 terminus. The peptide sequence is deduced by considering precise mass differences between adjacent y"-ions. (**D**) Immunoblotting of ELB 13/3-56 and control anti-COMP antibody against purified COMP: lane 1 control antibody against unreduced COMP; lane 2 ELB 13/3-56 against unreduced COMP; lane 3 control antibody against reduced COMP; lane 4 ELB 13/3-56 against reduced COMP.*

Cartilage oligomeric matrix protein (COMP) is a 524-kD protein expressed at high levels in the territorial matrix of chondrocytes. The sequences of rat and bovine COMP indicate that it is a member of the thrombospondin gene family mapped and in humans is localised in 19p13.1 gene [92]. COMP gene mutations are associated with human skeletal malformations as pseudoachondroplasia [93, 94] and one form of multiple epiphyseal dysplasia [93].

3.2.5. Potential epitope for ELB13/3-56 located at the C-terminal region of COMP

Subsequent to the identification of antigen-specificity of ELB13/3-56 we focused on the characterization of the respective epitope by the application of a mimotope strategy that was originally described by Geysen et al [95]. This approach is based on the fact that in some cases discontinuous epitopes on

proteins can be mimicked by short peptides. Hence peptides can be effectively used to define antibody specificity [96-99]. Therefore, purified ELB13/3-56 was immobilized on petri-dishes and repetitively panned against a heptameric phage library. With every round of panning there was an enrichment of sequences giving rise to specific recognition by ELB13/3-56. Table 6 lists the amino acid sequences of peptides expressed on phages binding capacity to ELB13/3-56. All sequences have been aligned and yield a consensus motif XSPPNVP. This consensus motif was found to be most closely related to the COMP sequence KDPRNVG (amino acid residues 669 – 675).

Sequence	Frequency	Biopanning Round
NQD**V**PLF	4	1st (1) and 2nd (3)
TL**P**LY**V**P	1	1st
T**K**S**P**P**N**Q	1	1st
YS**P**P**NV**P	8	1st (1) and 2nd (7)
XS**P**P**NV**P	Consensus Motif	
KD**P**R**NV**G	COMP	

***Table 6**- Aligned amino acid (a.a) sequences of peptides expressed on phages binding to ELB13/3-56 and compared to the COMP sequence. Bold a.a. indicate homology to the COMP sequence; underlined a.a. indicate homology to the consensus motif.*

3.2.6. Heptameric COMP-epitope binds efficiently RA serum

The identification of a heptameric COMP-peptide as an epitope of a human synovial B cell hybridoma led us to determine whether its recognition

by IgG autoantibodies is possibly disease-specific. Thus, serum samples of RA (n=22) and OA (=24) patients and from healthy donors (n=20) were tested on ELISA-plates coated with the COMP heptamer. As depicted in figure 16A the serum samples taken from RA patients bound with significantly higher efficiency to the COMP heptamer than the OA sera ($p<1x10^{-4}$, Students t-test) or the healthy controls ($p<1x10^{-4}$, Students t-test) indicating a disease specific autoantibody response. This disease specific response was confirmed by the more efficient binding of ELB 13/3-56 to the COMP heptamer than to the control on (Fig 16B).

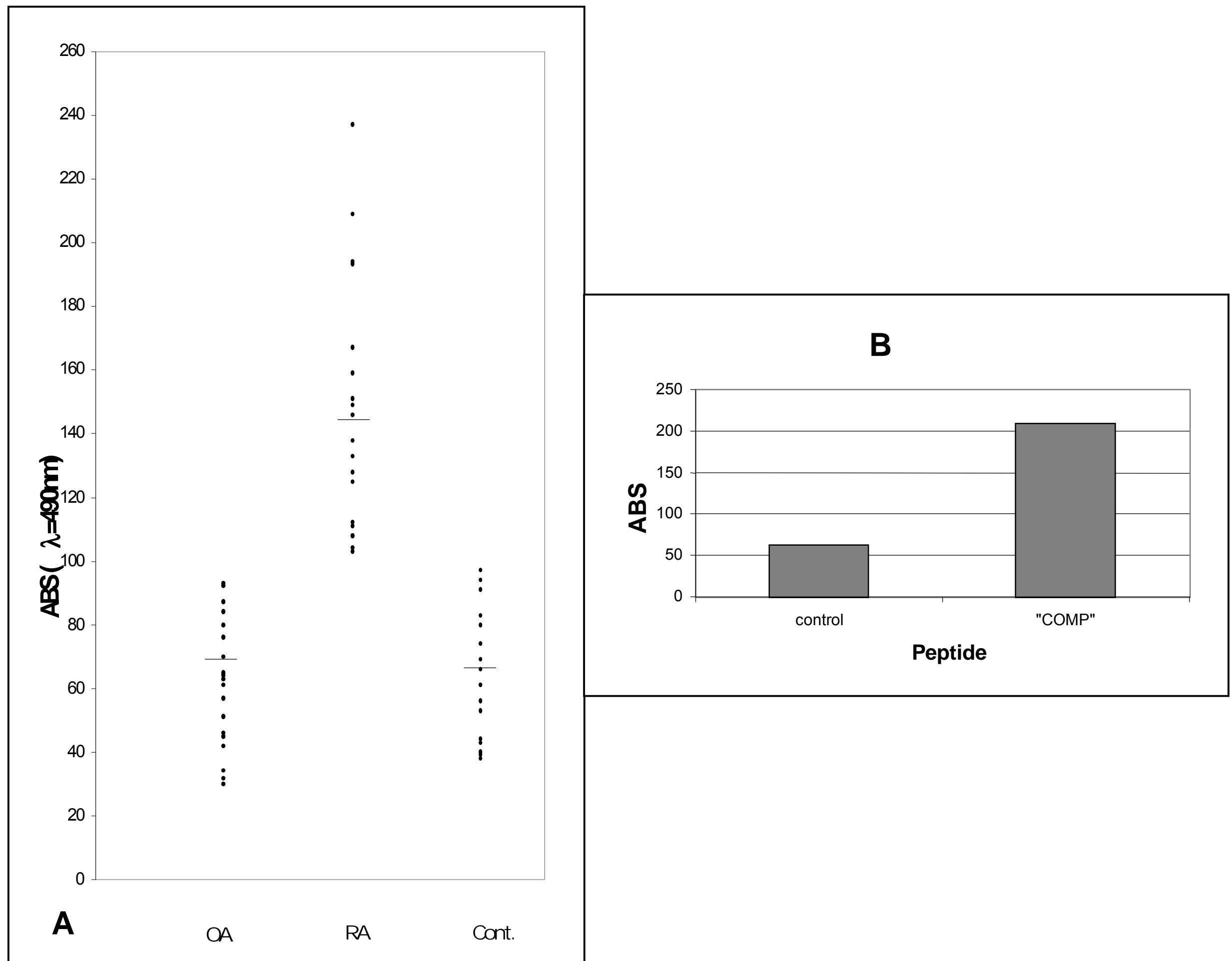

***Figure 16**- IgG extinction values at λ= 490nm x10^3 of the peptid ELISA: **A**) from human sera of OA patients (n=24), RA patients (n=22) and age matched*

healthy controls (n=22). Mean values OA= 69.25, RA= 142.41 and healthy controls= 66.13. ***B****) from ELB 13/3-56 with the control and the potential COMP-epitope. All data points are the mean of 4 independent experiments.*

4. DISCUSSION

4.1. IgVH genes from different anatomical regions -with different histopathological patterns- of a rheumatoid arthritis patient suggest cyclic re-entry of mature synovial B-cells in the hypermutation process

Various studies have demonstrated that somatically mutated B-cells are present in rheumatoid arthritis synovial tissue [26, 28, 54-56] and in human rheumatoid arthritis hybridomas [21, 57]. However for the first time we studied the IgV genes of synovial B-cells taken from different anatomical regions -having distinct histopathology and local disease duration- of the same rheumatoid arthritis patient. The analysis of the 55 IgVH sequences corroborates the findings of other groups that studied a single location and adds further information on B-cell distribution and activation in rheumatoid arthritis.

4.1.1. Amino acid deletions and mixed molecules: A novel pathway to generate antibody specificities?

Recently the introduction of deletions and duplications in addition to nucleotide exchanges has been described as features from the somatic hypermutation process [80, 100]. However amino acid deletions in the IgV genes have only been described in lymphomas and healthy secondary lymphatic tissue [80, 100]. In the present study we report the existence of such amino acid deletions in the IgV genes from synovial B-lymphocytes of an

autoimmune disease. For the first time, amino acid deletions in the VH genes were found in B-cells of an autoimmune disease. The detection of these deletion events in rheumatoid arthritis synovialitis stresses the functional homology of the synovial membrane to secondary lymphatic tissue.

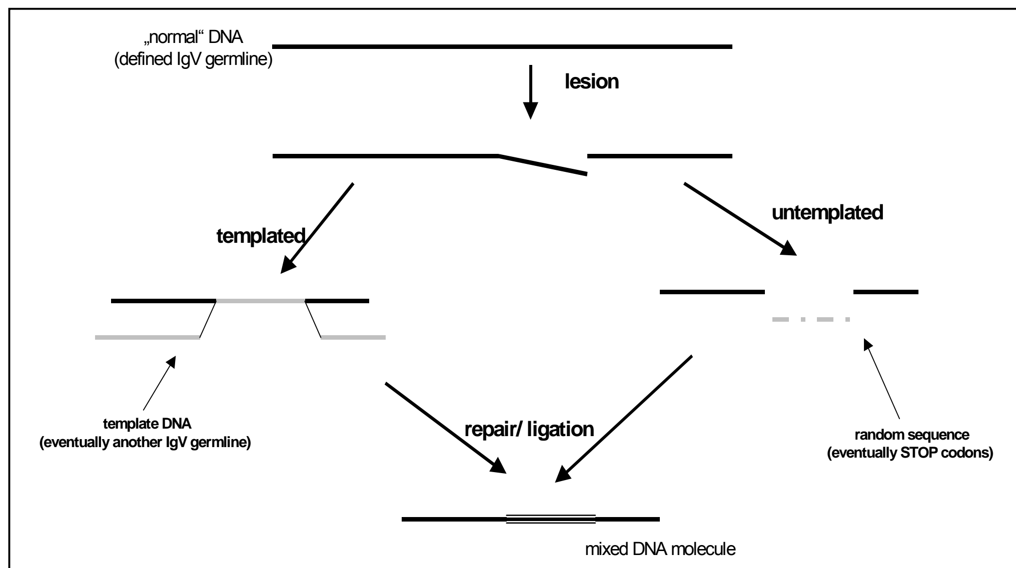

***Figure 17**- Generation of mixed molecules according to Maizels [98]: in rheumatoid arthritis synovial tissue mixed molecules could be the result of a templated or an untemplated hypermutation DNA repair process.*

Also for the first time we found IgV gene mixed molecules formed either by two segments of different IgV genes or by IgV gene rearrangement fragmented by random insertions. The mechanisms underlying the formation of these mixed molecules could be modified pathways to the unified model for somatic hypermutation proposed by Maizels [101] (fig 17): an initiating lesion could lead to a hypermutation either templated (using another germline gene as template) or untemplated (by inserting small random sequences). In some cases the process of untemplated hypermutation could lead to the insertion of STOP codons rendering the gene non-functional.

Hence the production of mixed molecules and the introduction of deletions could represent novel pathways for rheumatoid arthritis synovial B-cells to generate new specificities leading, for instance, to autoreactive antibodies that could contribute to the local and systemic tissue destruction.

4.1.2. Apparent mutational pattern among the 19 amino acid VH1 segments

The comparison of the amino acid sequences of the 19 VH1 segments from the different localisations provided some valuable data for the understanding of the interaction of the rheumatoid arthritis synovial B-cells and their target antigen(s).

All of these VH1 sequences presented mainly conservative mutations in the FR and non-conservative in the CDR. Thus, they agree with the results from Wedemayer et al [102]. When solving at 2.1Å resolution the crystal structure of a germline antibody Fab fragment and its complex with hapten they observed an expansion of the binding potential of the primary antibody potential. This expansion derived from configurational stability due to antigen binding and somatic mutations, non-conservative mutations in the CDR raising the affinity for the hapten, and conservative mutations in the FR.

Another important finding was the almost overall conservation of the mutational cold spots and „structural cold spots" [73] among the 19 VH1 segments. The interesting absence of mutations in positions 17, 23, 28, 30, 48, 56 and 71 suggests the existence of more „structural cold spots„ in the VH1 family than the ones described.

During the germinal center reaction (for instance in the follicles of the two earlier lesions of this patient) rearranged B-cells with low affinity receptors improve their affinity by somatic hypermutation [103, 104]. Nevertheless, these mutations can also decrease the affinity instead of expanding it. Therefore, as reported by Meffre et al [105] under appropriate regulation VDJ rearrangements take place in mature B-cells of human tonsil. Hence, the heterogenity observed on the CDR3 of the 19 VH1 sequences could be due to a reactivation of the rearrangement process in order to rescue these rheumatoid arthritis synovial B-cells from deleterious somatic mutations, or to further increase their binding affinity.

Based on the above mentioned findings, there seems to be a mutational pattern among all these 19 VH1 segments, hence suggesting that in all three RA lesions of this patient the synovial B-cells were activated by a restricted number of antigens. This is strengthened by the replacement in position 34 of CDR2 that could be interpreted as an antigen-selected mutational hotspot.

4.1.3. Cyclic re-entry of mutated rheumatoid arthritis synovial B-cells in the hypermutation process

The increment of mutations with antigen dose [106] possibly indicates that the maturation of the immune response is a continuous process with the production of an increasing number of hypermutate memory B-cells with time. In the special case of rheumatoid arthritis the local joint destruction may release antigens that lead to the hypermutation process. Characteristic for B-

cell hypermutation are the elevated R/S ratios in the CDR. In our study there was a direct association of the overall R/S ratios with the time of local disease duration. Since synovial B-cells were shown to undergo a germinal center-like reaction in rheumatoid arthritis [26, 27, 54, 55, 100], we put forward that the observed activated B-cells may have undergone a local maturation in the germinal center-like structures detected in the two peroneal tendons. On the other hand, the fact that one lesion was free from of FDCs and exhibited only an acute inflammatory infiltrate could support the model proposed by Oprea et al [107]: the already mutated germinal center B-cells from the peroneal tendons may have migrated into the cubita synovial tissue –as shown for closely located finger joints [108] and re-entered in a cyclic hypermutation process. The apparent existence of a mutational pattern on amino acid level of clones obtained from the different regions could support this hypothesis. However, the existence of clonally related B-cells in the cubita and left peroneal tendon leaves no doubts to assume that in our patient there is a cyclic re-entry of the mutated B-cells from the early rheumatoid arthritis lesions in the hypermutation process [109] and also sequentially colonize new germinal centers, as proposed by Kepler et al [110].

4.2. Human rheumatoid synovial B-cell hybridoma with a new disease related specificity for Cartilage Oligomeric Matrix Protein (COMP)

The destruction of joint cartilage and tendon is a key feature of rheumatoid arthritis. Histopathology and the molecular analysis of lymphocyte receptors for clonality indicate that T- and B-cells are expanded in an antigen

dependent manner in the inflamed synovial tissue, and the inflammatory reaction is related to local disease activity [10]. However, antigens of pathogenic relevance, especially those of tissue-specificity (-arthritogenic antigens-), have not yet been identified. In the present study a RF negative human rheumatoid synovial B-cell hybridoma was characterised for the specific recognition of COMP, an extracellular matrix protein restricted in its expression to cartilage and tendons [90, 92]. Hence, this human COMP-specific antibody targets structures in the joint that are preferentially affected by the inflammatory process in rheumatoid arthritis and can therefore be considered a prototype of a tissue-specific autoantibody. It belongs to the IgG2 class and may directly cause tissue destruction due to its complement fixing capacity.

4.2.1. Fine-specificity of the COMP specific B-cell hybridoma

The success of the epitope mapping strategy for the ELB13/3-56 mAb in the present study stresses the usefulness of phage libraries displaying small peptides as powerful tools to investigate antibody fine-specificities. However the delineation of a short peptide consensus sequences and the deduced identification of a heptameric COMP-epitope raises the question whether the identified motif represents the entire epitope, or rather a continuous part within a discontinuous determinant. X-ray analysis of antibody-antigen complexes by Barlow et al [111] estimated that most antibodies are probably raised against discontinuous determinants consisting of 15 to 22 residues on several surface loops. On the other hand, semiquantitative estimations of Gibbs free energy changes by Novotny et al

[112] have predicted that only few of the determinant residues contribute actively to the binding energetics, and the surrounding residues allow structural complementarity. Therefore, the obtained consensus motif possibly mimics a minimal epitope or parts of the epitope recognized by this hybridoma.

4.2.2. Local generation of a rheumatoid arthritis associated COMP specific autoantibody

In rheumatoid arthritis germinal center like structures are detectable in the inflamed synovial tissue suggestive for a locally generated B cell response to tissue-specific (auto)antigens [56, 113]. In this respect the characterization of a COMP specific synovial B-cell hybridoma provides first experimental evidence for the nature of the antigen(s) possibly involved. The B cell hybridoma is specific for a tendon- and cartilage-specific antigen and carries somatically mutated IgVH genes with high R/S values in the CDR regions as characteristics of an antigen-driven affinity maturation [30, 56].

Moreover, the clonotypic sequences of the COMP specific hybridoma could be detected in synovectomy material derived from different rheumatoid arthritis-patients but in none of the investigated OA cases. This indicates that the occurrence of B cells with somatically muted VH genes nearly identical to those coding for a COMP-specific antigen receptor are characteristic for the chronic synovitis of rheumatoid arthritis. Hence B-cell activation in rheumatoid arthritis and OA follow different patterns [61]. The vigor of the local immune response to COMP in the joints is also reflected by the detection of circulating

IgG autoantibodies with specificity for the identified heptameric COMP-determinant in the serum of rheumatoid arthritis-patients.

4.2.3. Arthritogenic immune response to COMP suggests a failure in tolerance induction

Various studies on serum, synovial tissue and synovia of rheumatoid arthritis patients detected the presence of antibodies to type I [114], type II [114, 115] and denatured collagen. This probably reflects an ongoing autoimmune response to cartilage proteins in rheumatoid arthritis. A similar scenario has been reproduced to certain extent on animal models such as type II collagen induced arthritis (CIA) in rats [116] and more recently in COMP-induced arthritis in rats [6].

In the COMP model the arthritis inducer is a homologous protein. Moreover, the response is MHC dependent and the autoantibodies are mainly of IgG type rather than IgM suggesting an important role of autoreactive T-cells [6].

From these facts one can draw some paralels to the immune response involving the present anti-COMP antibody: 1) as in the animal model ELB 13/3-56 is also an IgG autoantibody directed against a homologous protein. 2) Since MHC proteins are only capable of presenting peptides homologous to the structures in their binding pockets a search in the SYFPEITHI data base [117] revealed the existence of HLA-DRB1*0401 molecules –which are associated with RA- with a portion of their peptide binding pockets homologous to the potential COMP-epitope (Tab 7). In MHC class II

molecules as HLA-DRB1*0401 the ligands consist of 12 to 25 amino acids. However only nine occupy the binding groove, with between two and four anchored in the pockets and the other playing a secondary role [118].

Position								
1	2	3	**4**	5	**6**	**7**	8	**9**
F			**P**		**N**	**D**		**D**
Y			**W**		**S**	**E**		**E**
W			**I**		**T**	**H**		**H**
I			**L**		**Q**	**K**		**K**
L			**V**		**H**	**N**		**N**
V			**A**		**R**	**Q**		**Q**
M			**D**			**R**		**R**
			E			**S**		**S**
						T		**T**
						Y		**Y**
						A		**A**
						C		**C**
						I		**I**
						L		**L**
						M		**M**
						V		**V**
COMP-epitope								
		K	D	P	R	N	V	G

Table 7- *Search report obtained from the SYFPEITHI data base [117] for peptide motifs binding to HLA-DRB1*0401 molecules.* **Bold** *amino acids and position numbers indicate anchors in the pockets*; underlined *amino acids indicate homology to the potential COMP-eptitope.*

Taken together these facts lead to the conclusion that also in the autoimmune response to COMP in RA there is a marked importance of autoreactive T-cells. This raises the question why the immune system is not tolerized against

COMP. The question is even more pertinent when one takes under consideration that COMP is released from both normal and diseased joints.

One possible explanation could be the one suggested by Carlsen et al [6] that less abundant cartilage proteins such as COMP have a lower efficience in tolerance induction. Another explanation is that the circu;ating COMP molecules/fragments might expose other epitopes than the ones of intact COMP chains or of COMP incorporated in the extracellular cartilage matrix, thus allowing the COMP-autoreactive T-cells to escape from clonal deletion un the thymus.

4.2.4. Synovial autoantibody with a perpetuating function in joint destruction

As mentioned, further evidence for a potential role of COMP as an arthritogenic autoantigen in rheumatoid arthritis is derived from recent animal experiments. Thus, COMP has been shown to induce arthritogenic immune responses in rodents [6].

Autoimmunity to COMP could contribute to the pathogenesis of rheumatoid arthritis in keeping active the inflammatory process in the joints. In this respect synovial B cells could be crucial for the chronicity of the arthritic process in rheumatoid arthritis similar to their role in experimental disease models in rodents [119]. Especially the local production of complement fixing autoantibodies to cartilage components (e.g. the IgG2λ monospecific ELB13/3-56) like COMP could lead to a continuous immune complex formation with the cartilage matrix thereby attracting polymorphonuclear leukocytes and macrophages. The resulting engulfment of the immune

complexes is inevitably accompanied by proteolytic damage of the cartilage matrix.

4.3. Conclusion and future prespectives

Hitherto no organ-specific arthritogenic antigen responsible for T- and B-lymphocyte activation in rheumatoid arthritis has been described. One of the main reasons is the absence of a suitable animal model for this enigmatic arthritic disease. However, ignorance of the factors leading to initiation and perpetuation of rheumatoid arthritis also play a decisive role in slowing down the development of new successful therapeutical or diagnostical strategies for this condition.

In the present study rheumatoid synovial B-lymphocytes have been analysed in order to shed a new light into the role of these cells in the inflammatory process of rheumatoid arthritis. Two striking results have been obtained: 1st there is a recirculation of somatically mutated B-lymphocytes; 2nd one of these somatically mutated rheumatoid synovial B-lymphocytes was specific for COMP. Based on these findings the following possible model for rheumatoid arthritis is proposed (fig 18):

The work of Gregersen et al [120] postulated that an identical genetic element of the 3rd hypervariable region sequence shared by the HLA-DR4 and HLA-DR1 (depending on the ethnic origin of the studied population) molecules is likely to be the molecular basis of genetic susceptibility to rheumatoid arthritis. However, contrasting to the strong association between HLA-B27 and ankylosing spondylitis (individuals born with HLA-B27 are about 300 times more susceptible to develop ankylosing spondylitis than other [121]),

individuals having a HLA-DR4 or DR1 genetical background are only about 7 times more likely to develop rheumatoid arthritis [122]. Therefore, other factors leading to initiation of rheumatoid arthritis must be taken into consideration. For example, unspecific tissue injury due to sports or accidents may release antigens which otherwise would not be accessible to the immune-system. Another hypothesis is that a viral or bacterial infection may also be an initiating factor for rheumatoid arthritis: eventual mimicry between viral/ bacterial epitopes and joint components may result in a „redirection“ of the immune response from the virus/ bacteria towards the joint components. Despite all these possibilities the question about rheumatoid arthritis initiation remains unanswered.

Two important characteristics of rheumatoid arthritis are its chronicity and also the inflammatory relapses. This poses the question on the mechanisms underlying the perpetuation of the disease. According to figure 18 and based on the results of the present study, one could imagine that germinal center-like structures form in the synovial membrane of the first disease location (usually the interfalangial joints). In these structures a local B-lymphocyte maturation –including somatic hypermutation- takes place, resulting in new specificities against self-antigens. These activated auto-reactive B-lymphocytes may locally cyclic re-enter the hypermutation process, or by recirculation may invade other anatomical regions leading to formation of new germinal center-like structures in the new affected joint. Finally, the liberation of tissue-specific autoantigens (e.g. collagens, aggrecan or COMP) from the injured cartilage could fuel again the vicious cycle thereby leading to the perpetuation and amplification of rheumatoid arthritis joint inflammation.

Initiation?

HLA-DRB1^{++}
Unspecific Tissue Injury (sports, accidents)
Viral/ Bacterial Infection (Epitope Mimicry)
Psycological Issues

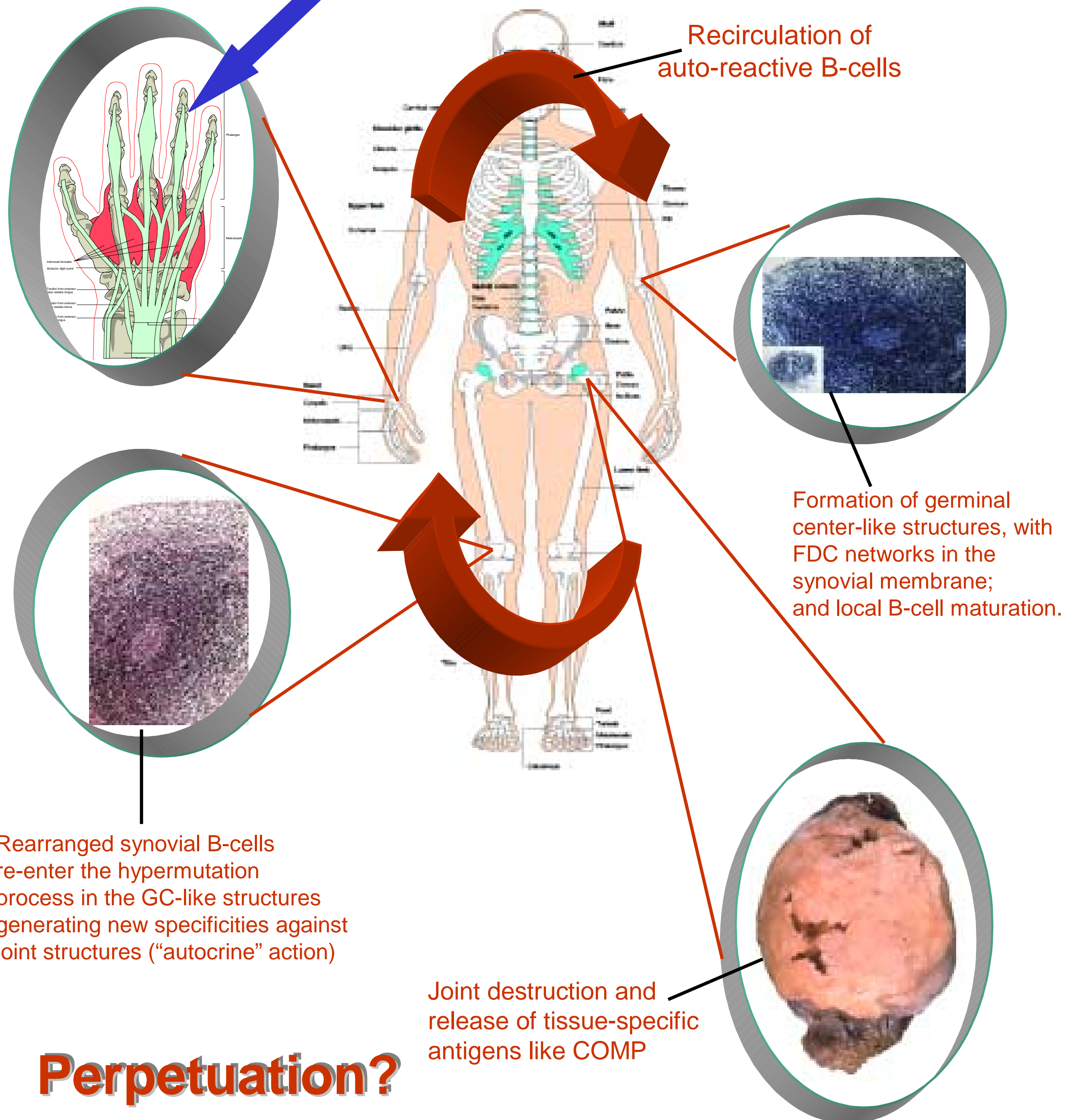

Figure 18- *Hypothetical model for the perpetuation of human rheumatoid arthritis inflammation and joint destruction.*

It is generally accepted that an early diagnosis of rheumatoid arthritis raises the prospectives of a more successful therapy, which does not lead to a total cure, but prevents it from disabling the patient to lead a normal life. In the present study there was a high specificity of the rheumatoid arthritis sera to the potential COMP-epitope. The present serological data for the COMP-epitope have been recentely compared to other parameters for rheumatoid arthritis obtained for the same group of patients (personal comunication from Dr. HG Kraetsch). The total lack of correlation between the binding to the COMP-epitope and collagen type II, extent of cartilage destruction or blood sedimentation suggest that we are in the presence of a new disease specific parameter. Eventhough this serological test has to be extended to a larger number of rheumatoid arthritis patients (with different disease stages) and also to other arthritic diseases, it is legitimate to speculate that the COMP-epitope could be the starting point for a new diagnostical strategy for rheumatoid arthritis.

5. LITERATURE

1. Firestein GS, Zvaifler NJ. 1992. Autoimmune arthropathy: rheumatoid synovitis. In Rose NR & Mackay IR (Ed) The autoimmune diseases II, Academic Press Inc/ USA: 363.
2. Brewerton D. 1995. All about arthritis: past, present, future. Harvard University Press/ USA: 91-98.
3. Kirchner H, Kruse A, Neustock P, Rink L. 1994. Cytokine und Interferone: Botenstoffe des Immunsystems. Spectrum Akademischer Verlag/ Germany: 185.
4. Waller E. (1940): On the occurence of a factor in human serum activating the specific agglutination of sheep corpuscles. Acta Pathol Microbiol Scand 17:172-188.
5. Sack U. 1997. Tiermodelle der rheumatoiden Arthritis. In U. Sack (Ed) Update: Clin Immunol 5: 256.
6. Stiehl P. (1997). Histologie der Rheumatoid-Arthritis. Beitrag zur diagnostischen und pathogenetischen Heterogenität, zur Aktivitätsdiagnose und Prognose. In U. Sack (Ed) Update: Clin Immunol 5: 188-200.
7. Carlsen S, Hansson AS, Olsson H, Heinegard D, Holmdahl R. 1998. Cartilage oligomeric matrix protein (COMP)-induced arthritis in rats. Clin Exp Immunol 114 (3), 477-484.
8. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR. 2000. Macrophages in rheumatoid arthritis. Arthritis Res 2 (3): 189-202.

9. Petrow PK, Bräuer R. 1997. T-Zellen und Arthritis. In U. Sack (Ed) Update: Clin Immunol 5: 300-315.
10. Fugger L, Svejgaard A. 2000. Association of MHC and rheumatoid arthritis. HLA-DR4 and rheumatoid arthritis: studies in mice and men. Arthritis Res 2 (3): 208-211.
11. Uematsu Y, Wege H, Straus A, Ott M, Bannwarth B, Lanchbury JSS, Panayi GS, Steinmetz TM. 1991. The T-cell-receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal. Proc Natl Acad Sci USA 88: 8534-8538.
12. Howell M, Diveley JP, Lundeen KA, Esty A, Winters ST, Carlo DJ, Brostoff SW. 1991. Limited T-cell-receptor b-chain heterogeneity among interleukin 2-receptor positive T-cells suggests a role for a superantigen in rheumatoid arthritis. Proc Natl Acad Sci USA 88: 10921-10925.
13. Paliard X, West SG, Lafferty JA, Clements JR, Kappler JW, Marrack JP, Kotzin BL. 1991. Evidence for the effects of a superantigen in rheumatoid arthritis. Science 253: 325-329.
14. Thomson W, Harrison B, Ollier B, Wiles N, Payton T, Barrett J, Symmons D, Silman A. 1999. Quantifying the exact role of HLA-DRB1 alleles in susceptibility to inflammatory polyarthritis: results from a large, population-cased study. Arthritis Rheum 42: 757-762.
15. Nepom GT. 1998. Major histocompatibility complex-directed susceptibility to rheumatoid arthritis. Adv Immunol 68: 315-332.
16. Roudier J. 2000. Association of MHC and rheumatoid arthritis. Association of RA with HLA-DR4: the role of repertoire selection. Arthritis Res 2 (3): 217-220.

17. Drakesmith H, Chain B, Beverley P. 2000. How can dendritic cells cause autoimmune disease? Immunol Today 21 (5): 214-217.
18. Krenn V, Vollmers HP, von Landenberg P, Schmaußer B, Rupp M, Roggenkamp A, Müller-Hermelink HK. (1996). Immortalized B-lymphocytes from rheumatoid synovial tissue show specificity for bacterial HSP 60. Virchows Arch 427: 511-518.
19. Randen I, Mellbye OJ, Forre O, Natvig JB. (1995). The identification of germinal centers and follicular dendritic cell networks in rheumatoid synovial tissue. Scand J Immunol 41: 481-486.
20. Berek C, Berger A, Apel M. (1991). Maturation of the immune response in germinal centers. Cell 67: 1121-1129.
21. Krenn V, König A, Hensel F, Berek C, Souto-Carneiro MM, Haedicke W, Wang YK, Vollmers HP, Müller-Hermelink HK. (1999). Molecular analysis of rheuma factor (RF)-negative B-cell hybridomas from rheumatoid synovial tissue: evidence for an antigen-induced stimulation with selection of high mutated IgVH and low mutated IgVL/ genes. Clin Exp Immunol 115: 168-175.
22. Miller C, Stedra J, Kelsoe G, Cerny J. (1995). Facultative role of germinal centers and T-cells in the somatic diversification of IgVH genes. J Exp Med 181: 1319-1331.
23. Camacho SA, Kosco-Villbois MH and Berek C. (1998). The dynamic structure of the germinal center. Immunol Today 19 (11): 511-514.
24. MacLennan IC. (1994). Germinal centers. Annu Rev Immunol 12: 117-139.

25. Wagner UG, Kurtin PJ, Wahner A, Brackertz M, Berry DJ, Goronzy JJ, Weynand CM. (1998). The role of $CD8^+$ $CD40L^+$ T-cells in the formation of germinal centers in rheumatoid synovitis. J Immunol 161: 6390-6397.
26. Schröder AE, Greiner A, Seyfert C, Berek C. (1996). Differentiation of B cells in the non-lymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. Proc Natl Acad Sci USA 93: 221-225.
27. Kim HJ, Krenn V, Steinhauser G, Berek C. (1999). Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis. J Immunol 162 (5): 3053-3062.
28. Krenn V, Molitoris R, Berek C, Sack U, König A, Müller-Deubert S, Kempf V, Mosgoeller W, Kerkau T, Vollmers HP, Müller-Hermelink HK. (1998). A novel monospecific IgG2/lambda-autoantibody with specificity for a mitochondrial antigen: Evidence for an antigen-driven pathogenetic B-cell response in rheumatoid synovial tissue, induced by tissue alteration. Lab Invest 79 (4): 485-496.
29. Shlomchik MJ, Marshak-Rothstein A, Wolfowicz CB, Rothstein TL, Weigert MG. (1987). The role of clonal selection and somatic mutation in autoimmunity. Nature 328: 805-811.
30. Chang B, Casali P. (1994). The CDR1 sequences of a major proportion of human germline IgVH genes are inherently susceptible to amino acid replacement. Immunol Today 15 (8): 367-373.
31. Burastero SE, Cutolo M, Dessi V, Celada F. (1990). Monoreactive and polyreactive rheumatoid factors produced by in vitro EBV transformed peripheral blood and synovial B-lymphocytes from rheumatoid patients. Scand J Immunol 32: 347-357.

32. Lu EW, Deftos M, Olee T, Huang D, Soto-Gil R, Carson D, Chen P. (1993). Generation and molecular analyses of two rheumatoid synovial fluid-derived IgG rheumatoid factors. Arthritis Rheum 36: 927-937.
33. Ezaki I, Kanda H, Sakai K, Fukui N, Shingu M, Nobunaga M, Watanabe T. (1991). Restricted diversity of the variable region nucleotide sequences of the heavy and light chains of a human rheumatoid factor. Arthritis Rheum 34: 343-341.
34. Randen I, Pascual V, Victor K, Thompson KM, Forre O, Capra JD, Natvig JB. (1993). Synovial IgG rheumatoid factors show evidence of an antigen-driven immune response and a shift in the V gene repertoire compared to IgM rheumatoid factors. Eur J Immunol 23: 1220-1225.
35. Borretzen M, Chapman C, Natvig JB, Thompson KM. (1997). Differences in mutational patterns between rheumatoid factors in health and disease are related to variable heavy chain family and germ-line gene usage. Eur J Immunol 27: 735-741.
36. Mulder A, Horst M, van Leeuwen A, Limburg C, Kallenberg G. (1993). Antineutrophil cytoplasmic antibodies in rheumatoid arthritis. Characterisation and clinical correlations. Arthritis Rheum 36: 1054-1062.
37. Emmrich F, Wang J. (1995). Frequencies of autoreactive collagen type II specific B-cells in rheumatoid arthritis. Scand J Rheumatol Suppl 101: 39-41.
38. Rudolphi U, Rzepka R, Batsford S, Kaufmann SH, van der Mark K, Peter HH, Melchers I. (1997). The B-cell repertoire of patients with rheumatoid arthritis. II. Increased frequencies of IgG+ and IgA+ B-cells specific for

mycobacterial heat-shock protein 60 or human type II collagen in synovial fluid and tissue. Arthritis Rheum 40 (8): 1409-1419.

39. Marnell L, Serals R, Savage M, Jaramillo Y, Sibitt W. (1990). L Anti-class II ß-chain antibodies in the serum of synovial fluid of rheumatoid arthritis patients. Arthritis Rheum 36: 229-232.

40. Randen I, Thompson KM, Natvig JB, Forre O, Waalen K. (1989). Human monoclonal rheumatoid factors derived from the polyclonal repertoire of rheumatoid synovial tissue: production and characterization. Clin Exp Immunol 78 (1): 13-18.

41. Schellenkes GA, de Jong BAW, van den Hoogen FHJ, van de Putte LBA. (1998). Citrulline is essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 101 (1): 273-281.

42. Shoenfeld Y. (1994). Idiotypic induction of autoimmunity: do we need an autoantigen? Clin Exp Rheumatol 12 Suppl 11: 37-40.

43. Hassan J, Yanni G, Hegarty V, Feighery C, Bresnihan B, Whelan A. (1996). Increased numbers of $CD5^+$ B cells and T cell receptor (TCR) gamma $delta^+$ T cells are associated with younger age of onset in rheumatoid arthritis (RA). Clin Exp Immunol 103 (3): 353-356.

44. Youinou PY, Morrow JW, Lettin AWF, Lydyard PM, Rioitt IM. (1984). Specificity of plasma cells in the rheumatoid synovium. Scand J Immunol 20: 307-315.

45. Zvaifler NJ. (1973). The immunopathology of joint inflammation in rheumatoid arthritis. Adv Immunol 13: 265-336.

46. Sutton B, Corper A, Bonagura V, Taussig M. 2000. The structure and origin of rheumatoid factors. Immunol Today 21 (4): 177-183.
47. Olee T, Lu EW, Huang DF, Soto-Gil RW, Deftos M, Kozin F, Carson DA, Chen PP. (1992). Genetic analysis of self-associating immunoglobulin G rheumatoid factors from two rheumatoid synovia implicates an antigen-driven response. J Exp Med 175: 831-842.
48. Terato K, Hasty KA, Reife RA, Cremer MA, Kang AH, Stuart JM. (1992). Induction of arthritis with monoclonal antibodies to collagen. J Immunol 148 (7): 2103-2108.
49. Burkhardt H, Tan Y, Bröker B, Beck-Sickinger A, Holmdahl A, van der Mark K, Emmrich F. (1992). Antibody binding to a collagen type 2 eptitope gives rise to an inhibitory peptide for autoreactive T cells. Eur J Immunol 22: 1063-1067.
50. De Graeff-Meeder ER, van der Zee R, Rijkers GT, Schuurman HJ, Kuis W, Bijlsma JW, Zegers BJ, van Eden W. (1991). Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. Lancet 337 (8754): 1368-1372.
51. Jones DB, Coulson AF, Duff GW. (1993). Sequence homologies between hsp60 and autoantigens. Immunol Today 14 (3): 115-118.
52. De Graeff-Meeder ER, Voorhorst M, van Eden W, Schuurman HJ, Huber J, Barkley D, Maini RN, Kuis W, Rijkers GT, Zegers BJ. (1990). Antibodies to the mycobacterial 65-kD heat-shock protein are reactive with synovial tissue of adjuvant arthritic rats and patients with rheumatoid arthritis and osteoarthritis. Am J Pathol 137 (5): 1013-1017.

53. Kowal C, Weinstein A, Diamond B. (1999). Molecular mimicry between bacterial and self antigen in a patient with systemic lupus erythematosus. Eur J Immunol 29: 1901-1911.

54. Williams DG and Taylor PC. Clonal analysis of immunoglobulin mRNA in rheumatoid arthritis synovium: characterization of expanded IgG3 populations. Eur J Immunol 1997; 27: 476-485.

55. Gause A, Gundlach K, Zdichavsky M, Jacobs G, Koch B, Hopf T and Pfreundschuh M. The B-lymphocyte in rheumatoid arthritis: analysis of rearranged Vא genes from B cells infiltrating the synovial membrane. Eur J Immunol 1995; 25: 2775-2782.

56. Berek C and Kim HJ. B-cell activation and development within chronically inflamed synovium in rheumatoid and reactive arthritis. Immunol 1997; 9: 261-268.

57. Randen I, Brown D, Thompson KM, et al. Clonally related IgM rheumatoid factors undergo affinity maturation in the rheumatoid synovial tissue. J Immunol 1992, 148 (10): 3296-3301.

58. Arnett F, Edworthy S, Bloch D, *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; 31: 315-324.

59. Fuchs HA, Brooks RH, Callahan LF, Pincus T. A simplified twenty-eight-joint quantitative articular index in rheumatoid arthritis. Arthritis Rheum 1989; 32: 531-537.

60. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, Christy W, Cooke TD, Greenwald R, Hochberg M, et al. 1986. Development of criteria for the classification and reporting of osteoarthritis. Classification of

osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheuma 29 (8), 1039-1049.

61. Krenn V, Hensel F, Kim HJ, Souto-Carneiro MM, Ristow G, König A, Vollmers HP, Müller-Hermelink HK. (1999). Molecular IgV_H analysis demonstrates highly somatic mutated B-cells in synovialitis of osteoarthritis: A degenerative disease is associated with a specific, not locally generated immune response. Lab Invest 79(11): 1377-84.
62. Souto-Carneiro MM, Krenn V, Hermann R, König A, Müller-Hermelink HK. 2000. IgVH genes from different anatomical regions, with different histopathological patterns, of a rheumatoid arthritis patient suggest cyclic re-entry of mature B-cells in the hypermutation process. Arthritis Res 2 (4): in press.
63. Lefranc MP, Giudicelli V, Ginestoux C, et al. IMGT, the international ImMunoGeneTics database. Nucleic Acids Res 1999, 27(1):209-212
64. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (259), 680-685.
65. Towbin H, Staehelin T, Gordon J. 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology 24, 145-149.
66. Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68 (5), 850-858.
67. Müller EC, Schumann M, Rickers A, Bommert, K, Wittmann-Liebold B, Otto A. 1999. Study of Burkitt lymphoma cell line proteins by high

resolution two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. Electrophoresis 20, 320-330.

68. Otto A, Thiede B, Müller EC, Scheler C, Wittmann-Liebold B, Jungblut P. 1996. Identification of human myocardial proteins separated by two-dimensional electrophoresis using an effective sample preparation for mass spectrometry. Electrophoresis 17, 1643-1650.
69. Mann M, Wilm MS. 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal Chem. 66, 4390-4399.
70. Hedbom E, Antonsson P, Hjerpe A, Aeschlimann D, Paulsson M, Rosa-Pimentel E, Sommarin Y, Wendel M, Oldberg A, Heinegard D. 1992. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J Biol Chem 267 (9): 6132-6136
71. Küppers R, Zhao M, Rajewsky K, Hansmann ML. 1995. Detection of clonal B cell populations in paraffin-embedded tissues by polymerase chain reaction. Am J Pathol 143 (1): 230-239.
72. Dunn-Walters DK, Dogan A, Boursier L, MacDonald CM, Spencer J. Base-specific sequences that bias somatic hypermutation deduced by analysis of out-of-frame human IgVH genes. J Immunol 1998, 160: 2360-2364.
73. Dörner T, Brezinschek HP, Brezinschek RI, Foster SJ, Domiati-Saad R, Lipsky PE. Analysis of the frequency and pattern of somatic mutations within nonproductively rearranged human variable heavy chain genes. J Immunol 1997, 158: 2779-2789.

74. Dörner T, Brezinschek HP, Foster SJ, Brezinschek RI, Farner NL, Lipsky PE. Delineation of selective influences shaping the mutated expressed human Ig heavy chain repertoire. J Immunol 1998, 160: 2831-2841.
75. Drapkin R, Sancar A, Reinberg D. Where transcription meets repair. Cell 1994, 77: 9-12.
76. Neuberger MS, Milstein C. Somatic hypermutation. Curr Opin Immunol 1995; 7: 248-254.
77. Smith DS, Creadon G, Jena PK, Portanova JP, Kotzin BL, Wysocki LJ. Di- and trinucleotide target preferences of somatic mutagenesis in normal and autoreactive B cells. J Immunol 1996; 156: 2642-2652.
78. Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G. The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol 1992; 227: 776-798.
79. Betz AG, Neuberger MS, Milstein C. Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes. Immunol Today 1993; 14 (8): 405-411.
80. Wilson PC, de Bouteiler O, Liu Yj, et al. Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. J Exp Med 1998; 187 (1): 59-70.
81. Bridges SL Jr, Lee SK, Johnson ML, et al. Somatic mutation and CDR3 lengths in immunoglobulin kappa light chains expressed in patients with rheumatoid arthritis and in normal individuals. J Clin Invest 1995, 96 (2): 831-841.

82. Ebeling SB, Schutte MEM, Logtenberg T. Peripheral human CD5+ and CD5- B-cells may express somatically mutated Vh5 and Vh6 encoded IgM receptors. J Immunol 1993, 151: 6891-6899.
83. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin µ locus: characterisation of embrionic and rearranged J and D genes. Cell 1981; 27: 583.
84. Krenn V, Souto-Carneiro MM, KimHJ, Berek C, Starostik P, König A, Harms H, Müller-Hermelink HK. Histopathology and molecular pathology of synovial B-lymphocytes in rheumatoid arthritis. Histol Histopath 2000, in press.
85. Souto-Carneiro MM, Krenn V, Hermann R, Ristow G, König A, Müller-Hermelink HK (1999): Mutational pattern in the VH-genes fom B-cells taken from different anatomical locations from a rheumatoid arthritis patient. Verh Deut Ges Pathol: 83: 260-269.
86. Kristiansen SV, Pascual V, Lipsky PE. Straphylococcal protein A induces biased production of Ig by VH3-expressing B lymphocytes. J Immunol 1994; 153: 2974-2982
87. Hillson JL, Karr NS, Oppliger IR, Mannik M, Sasso EH. The structural basis of germline-encoded VH3 immunoglobulin binding to staphylococcal protein A. J Exp Med 1993, 178: 331-336.
88. Leippe M. Ancient weapons: NK-lysin, is a mammalian homolog ton pore-forming peptides of a protozoan parasite. Cell 1995, 83: 17-18.
89. Domiati-Saad R, Lipsky PE. Staphylococcal enterotoxin A induces survival of VH3-expressing human B-cells by binding to the Vh region with low affinity. J Immunol 1998, 161: 1257-1266.

90. DiCesare PE, Mörgelin M, Carlson CS, Pasumarti S, Paulsson M. 1995. Cartilage oligomeric matrix protein: isolation and characterization from human articular cartilage. J Orthop Res 13(3), 422-428.
91. Neidhart M, Hauser N, Paulsson M, DiCesare PE, Michel BA, Häuselmann HJ. 1997. Small fragments of cartilage oligomeric matrix protein in synovial fluid and serum as markers for cartilage degradation. Br J Rheumatol 36, 1151-1160.
92. Newton G, Weremowicz S, Morton C, Copeland N, Gilbert D, Jenkins N, Lawler J. 1994. Characterisation of human and mouse cartilage oligomeric matrix protein. Genomics 24: 435-439.
93. Briggs MD, Mortier GR, Cole WG, King LM, Golik SS, Bonaventure J, Nuytinck L, De Paepe A, Leroy JG, Biesecker L, Lipson M, Wilcox WR, Lachman RS, Rimoin DL, Knowlton RG, Cohn DH. 1998. Diverse mutations in the gene for cartilage oligomeric matrix protein in the pseudoachondroplasia-multiple epiphyseal dysplasia disease spectrum. Am J Hum Genet 62: 311-319.
94. Susic S, McGrory J, Ahier J, Cole WG. 1997. Multiple epiphyseal dysplasia and pseudoachondroplasia due to novel mutations in the calmodulin-like repeats of cartilage oligomeric matrix protein. Clin Genet 51: 219-224.
95. Geysen HM, Meloen RH, Barteling SJ. 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc Nat Acad Sci USA 81, 3998-4002
96. Scott JK, Smith GP. 1990. Searching for peptide ligands with an epitope library. Science 249, 386-390.

97. Cwirla SE, Peters EA, Barrett RW, Dower WJ. 1990. Peptides on phage: a vast library of peptides for identifying ligands. Proc Nat Acad Sci USA 87, 6378-6382.
98. Pinilla C, Martin R, Gran B, appel JR, Boggiano C, Wilson DB, Houghten RA. 1999. Exploring immunological specificity using synthetic peptide combinatorial libraries. Curr Opin Immunol 11, 193-202.
99. Böttger V, Böttger A, Lane EB, Spruce BA. 1995. Comprehensive epitope analysis of monoclonal anti-proenkephalin antibodies using phage display libraries and synthetic peptides: revelation of antibody fine specificities caused by somatic mutations in the variable region genes. J Mol Biol 247 (5), 932-946.
100. Goossens T, Klein U, Küppers R. Frequent occurence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci USA 1998; 95: 2463-2468.
101. Maizels N. Somatic hypermutation: how many mechanisms diversify V region sequences? Cell 1995, 83: 9-12.
102. Wedemayer GJ, Patten PA, Wang LH, Schultz pG, Stevens RC. Structural insights into the evolution of an antibody combining site. Science 1997, 276: 1665-1669.
103. Berek C, Milstein C. Mutation drift and repertoire shift in the maturation of immune response. Immunol Rev 1987; 96:23-41.
104. Berek C, Ziegner M. The maturation of the immune response. Immunol Today 1993; 14 (8): 400-404.

105. Meffre E, Papavasiliou F, Cohen P, et al. Antigen receptor engagement turns off the VDJ recombination machinery in human tonsil B cells. J Exp Med 1998, 188: 765-772.

106. González-Fernández A, Milstein C. low antigen dose favours selection of somatic mutants with hallmarks of antibody affinity maturation. Immunology 1998, 93:149-153.

107. Oprea M, Perelson AS. Somatic mutation leads to efficient affinity maturation when centrocytes recycle back to centroblasts. J Immunol 1997, 158: 5155-5162.

108. Voswinkel J, Weisgerber K, Pfreundschuh M, Gause A. The B-lymphocyte in rheumatoid arthritis: recirculation of B-lymphocytes between different joints and blood. Autoimmunity 1999, 31(1): 25-34.

109. Rajesky K. Clonal selection and learning in the antibody system. Nature 1996, 381: 751-758.

110. Kepler TB, Perelson AS. Cyclic re-entry of germinal center B-cells and the efficiency of affinity maturation. Immunol Today 1993, 14(8): 412-415.

111. Barlow DJ, Edwards MS, Thornton JM. 1986. Continuous and discontinuous protein antigenic determinants. Nature 322 (6081), 747-748.

112. Novotny J, Bruccoleri RE, Saul FA. 1989. On the attribution of binding energy in antigen-antibody complexes McPC 603, D1.3, and HyHEL-5. Biochemistry 28 (11), 4735-4749.

113. Neuberger MS, Milstein C. 1995. Somatic hypermutation. Curr Opin Immunol 7: 248-254.

114. Tarkowski A, Klareskog L, Carlsten H, Herberts P, Koopman WJ. 1989. Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. Arthritis Rheum 32: 1087-1092.

115. Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. 1996. Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression. Arthritis Rheum 39: 1720-1727.

116. Trentham DE, Townes AS, Kang AH. 1977. Autoimmunity to type II collagen an experimental model of arthritis. J Exp Med 146 (3): 857-868.

117. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Access via : http://www.uni-tuebingen.de/uni/kxi/

118. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50(3-4): 213-219.

119. Holmdahl R, Vingsbo C, Mo JA, Michaelsson E, Malmström V, Jansson L, Brunsberg U. 1995. Chronicity of tissue-specific experimental autoimmune disease: a role for B-cells? Immunol Rev 144: 109-135.

120. Gao XJ, Olsen NJ, Pincus T, Stastny P. 1990. HLA-DR alleles with naturally occuring amino acid substitution and risk for development of rheumatoid arthritis. Arthritis Rheum 33(7): 939-946.

121. Gregersen PK, Silver J, Winchester RJ. 1988. Genetic susceptibility to rheumatoid arthritis and human leukocyte antigen class II polymorphism: The role of shared conformational determinants. Am J Med 85(6A): 17-19.

122. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. 1973. Ankylosing spondylitis and HL-A27. Lancet 1(7804): 904-907.

6. ABSTRACT/ ZUSAMMENFASSUNG

6.1. English

B-cells of the rheumatoid synovial tissue are a constant part of and, in some histopathological subtypes, the dominant population of the inflammatory infiltrate, located in the region of tissue destruction. The pattern of B-cell distribution and the relationship to the corresponding antigen-presenting cells (follicular dendritic reticulum cells: FDCs) show a great variety. B-cells may exhibit (i) a follicular organization forming secondary follicles; (ii) follicle-like patterns with irregularly formed FDC networks, and (iii) a diffuse pattern of isolated FDCs. Molecular analysis of immunoglobulin VH and VL genes from human synovial B-cell hybridomas and synovial tissue demonstrates somatic mutations due to antigen activation. The FDC formations in the synovial tissue may therefore serve as an environment for B-cell maturation, which is involved in the generation of autoantibodies. An autoantibody is defined as "pathogenic" if it fulfills the Witebsky-Rose-Koch criteria for classical autoimmune diseases: definition of the autoantibody; induction of the disease by transfer of the autoantibody; and isolation of the autoantibody from the disease-specific lesion. B-cells from rheumatoid synovial tissue show specificity for FcIgG, type II collagen, COMP, sDNA, tetanus toxoid, mitochondrial antigens (M2), filaggrin and bacterial HSPs. The contributions of these antigens to the pathogenesis of RA are still hypothetical. A possible contribution could derive from crossreactivity and epitope mimicry: due to crossreaction, an antibody directed originally against a foreign infectious agent could react with epitopes from articular tissues, perpetuating the local inflammatory process. The characteristic distribution pattern, the localisation

within the area of tissue destruction, the hypermutated IgVH and IgVL genes, and their exclusive function to recognize conformation-dependent antigens suggest a central role for B-cells in the inflammatory process of rheumatoid arthritis. Therefore, the analysis of synovial B-cell hybridomas and experimental expression of synovial IgVH and IgVL genes will help to characterise the antigens responsible for the pathogenesis of rheumatoid arthritis.

In the present study 55 IgVH genes amplified from 3 different anatomical regions of a RA patient were analysed adding further information on synovial B-cell maturation and recirculation in RA.

This analysis demonstrated somatically mutated IgVh genes in all different regions with amino acid deletions and mixed IgVh molecules, suggesting the existence of a novel pathway to generate (auto)antibody specificities.

The comparison of amino acid sequences of amplified genes belonging to the VH1 family (with predominantly the same germline counterpart) exhibited a strong homology, indicating an apparently conserved mutational pattern. This suggests that the number of antigens activating B-cells in the different locations is restricted.

The most striking result was the finding of clonally related sequences in different anatomical regions indicating a recirculation of activated B-cells between the different affected joints.

Also in the present study a synovial B-cell hybridoma was analyzed for its specific recognition of cartilage antigens. A heptameric peptide of cartilage oligomeric protein (COMP) could be defined as the target structure. The IgVH-

gene (IgHV4-59*01) of the IgG2λ hybridoma has somatically mutated genes with high R/S values in the CDR regions (9:2). Thus, indicating that this hybridoma originates from a synovial B-cell which has been antigen activated/selected for its affinity. To analyse the presence of the clonotypic IgHV4-59*01 sequences in other cases of RA and osteoarthritis (OA) synovitis, primers specific for the CDR3 rearrangement of this hybridoma were used. The clonotypic and clone related sequences (98%±1% homology) could only be detected in synovitis of RA cases but not in OA cases indicating that this B-cell is specific to RA synovitis. The identified heptameric peptide of COMP was used in a peptide ELISA to analyse whether there is a specific binding in RA serum samples. Serum samples (IgG) from RA patients (n=22) showed a significant higher efficiency to the COMP heptamer than the OA sera (n=24) and the age matched healthy controls (n=20) (for both $p<1x10^{-4}$, Students t-test). The specificity of this B-cell hybridoma may therefore be defined as RA specific. Since COMP is restricted to cartilage and tendons which are organs specifically affected in RA this COMP specific autoantibody represents the first organ specific autoantibody in RA. The IgG2 COMP specific autoantibody with somatically mutated IgVH genes is different from germline encoded, antigen clearing IgM autoantibodies and may therefore be directly involved as an “arthritogenic autoantibody” in cartilage and tendons destruction by complement activation.

6.2. Deutsch

B-Zellen des rheumatoiden Synovialgewebes sind einerseits ein konstanter Bestandteil und andererseits in einigen histopathologischen Subtypen sogar die dominante Bevölkerung des entzündlichen Infiltrates, welches sich in direkter Nähe des zerstörten Gewebes befindet.

Das Strickmuster der B-Zellen-Verbreitung und die Verbindung zu den korrespondierenden antigenerzeugenden Zellen (follikuläre dendritische Zellen, sprich: FDC) zeigen eine große Vielfalt. B-Zellen können a) in der Form eines follikulären Verbandes sich bildender Sekundärfollikel; b) als follikelähnliche Muster mit unregelmäßig geformten FDC-Netzwerken und c) als ein diffuses Muster isolierter FDCs auftreten. Molekularanalysen der immunglobulinen VH- und VL-Gene von menschlichen synovialen B-Zell-Hybridomen und Synovialgewebe zeigen somatische Mutationen aufgrund von Antigenaktivierung auf. Die FDC-Schichten im Synovialgewebe dürften daher als ein Nährboden für B-Zell-Reifung dienen, welche wiederum in den Prozeß der Autoantikörperbildung eingreift. Ein Autoantikörper wird als „pathogenisch" bezeichnet, wenn er das Witebsky-Rose-Koch-Kriterium für klassische Autoimmunkrankheiten erfüllt: Bildung des Autoantikörpers; Einleitung der Krankheit durch Übertragung des Autoantikörpers und Isolation des Autoantikörpers vom krankheitsspezifischen Entzündungskomplex. B-Zellen aus rheumatoidem Synovialgewebe zeigen Spezifität für die Fc-Region von IgG; Typ II Kollagen; COMP; sDNA; Tetanustoxin; mitochondrische Antigene (M2); Fillaggrin und bakterielle HSPs. Der Beitrag dieser Antigene zur Pathogenese in der RA ist weiterhin hypothetisch. Ein möglicher Beitrag könnte aus der Kreuzreaktion und Epitopähnlichkeit aufgrund dieser

Kreuzreaktion herrühren. Ein Antikörper, der ursprünglich gegen einen fremden Infektionsherd gerichtet war, könnte mit Epitopen aus Gelenkgewebe reagieren und somit den lokalen Entzündungsprozeß aufrechterhalten. Das charakteristische Verteilungsmuster, die Lokalisierung inmitten des zerstörten Gewebes, die hypermutierten IgVH- und IgVL-Gene und ihre ausschließliche Funktion, strukturabhängige Antigene zu erkennen, läßt auf eine zentrale Bedeutung der B-Zellen im Bezug auf den Entzündungsverlauf in der rheumatoiden Arthritis schließen. Deswegen wird die Analyse synovialer B-Zellen-Hybridome und die experimentelle Erkundung synovialer IgVH- und IgVL-Gene eine große Hilfe zur Charakterisierung der Antigene sein, welche verantwortlich für die Pathogenese in der RA sind.

In dieser Studie wurden 55 IgVH-Gene analysiert, die von 3 verschiedenen anatomischen Regionen eines RA-Patienten amplifiziert wurden, wodurch man zusätzliche Informationen über synoviale B-Zellen-Reifung und –Rezirkulation in der RA erhielt. Diese Analyse zeigte somatisch mutierte IgVH-Gene in allen verschiedenen Regionen auf, mit Aminosäuredeletionen und gemischten IgVH-Molekülen, die Grund zur Annahme geben, daß eine neue Möglichkeit existiert, (Auto-) Antikörperspezifizierungen zu generieren. Der Vergleich von Aminosäuresequenzen amplifizierter Gene, die zur VH1-Familie gehören (mit überwiegend denselben Keimbahngenen) zeigten eine starke Homologie auf und wiesen auf ein scheinbar erhaltenes Mutationsmuster hin. Dieses läßt annehmen, daß die Anzahl der Antigene begrenzt ist, die B-Zellen in den verschiedenen Regionen aktivieren. Das markanteste Ergebnis war das Auffinden klonal verwandter Sequenzen in verschiedenen anatomischen

Regionen, die darauf hinweisen, daß aktivierte B-Zellen zwischen den verschiedenen befallenen Gelenken rezirkulieren.

Desweiteren wurde in dieser Studie ein synoviales B-Zellen-Hybridom analysiert bezüglich seiner spezifischen Erkennung von Knorpelantigenen. Ein heptameres Peptid des COMP könnte als eine mögliche Zielstruktur definiert werden. Die IgVH-Gene (IgHV4-59*01) des IgG2λ-Hybridomes besitzt somatisch mutierte Gene mit hohen R/S-Werten in den CDR-Regionen (9.2). Somit weist dies darauf hin, daß dieses Hybridom aus einer synovialen B-Zelle stammt, welche aufgrund ihrer Affinität antigen-aktiviert wurde. Um das Vorkommen der klonotypischen IgHV4-59*01-Sequenzen in anderen Fällen der RA und Osteoarthritis (OA)-Synovitis zu analysieren, wurden Primer benutzt, die spezifisch für die CDR3 dieses Hybridomes sind. Die klonotypischen und klonal verwandten Sequenzen (98%±1% Homologie) konnte nur in Fällen der RA-Synovitis erkannt werden, jedoch nicht bei OA-Fällen, was darauf hinweist, daß diese B-Zelle spezifisch für die RA-Synovitis ist. Das identifizierte heptamere COMP-Peptid wurde in einer Peptid-ELISA verwendet, um festzustellen, ob es eine spezifische Bindung in RA-Seren gibt. Seren (IgG) von RA-Patienten (n=22) zeigten eine bedeutend höhere Wirksamkeit des COMP-Heptamers an als in OA-Seren (n=24) und den altersabhängigen gesunden Kontrollen (n=20); (für beide $p<1x10^{-4}$; Students t-Test). Die Spezifizierung dieses B-Zellen-Hybridomes könnte daher als RA-spezifisch angesehen werden. Da COMP sich auf Knorpel und Sehnen beschränkt – welches hauptsächlich in der RA betroffene Organe sind – repräsentiert dieser COMP-spezifische Autoantikörper den ersten organspezifischen Autoantikörper in der RA. Der IgG2-COMP-spezifische

Autoantikörper mit somatisch mutierten IgVH-Genen unterscheidet sich von dem der antigenvernichtenden IgM Autoantikörper und könnte daher direkt in die Knorpel- und Sehnenzerstörung durch Komplementaktivierung miteinbezogen sein, als ein „arthritogener Autoantikörper“.

7. ABREVIATIONS

a.a. amino acid

APS Ammoniumpersulfate

bp base pairs

BSA bovine serum albumin

CD cluster of differentiation

cDNA complementary DNA

CDR complementarity determining region

CIA collagen induced arthritis

COMP cartilage oilgomeric matrix protein

DEPC Diethylpyrocarbonate

DMSO Dimethylsulfoxide

dNTP Deoxy Nukleotid Triphosphate

DTT di-Thiotreithol

EBV Epstein-Barr Virus

E. coli Escherichia coli

EDTA Ethylendiamintetra-acetate

ELISA enzyme linked immunosorbent assay

FasL Fas ligand

Fc constant fragment of a immunoglobulin molecule

FDC follicular dendritic cell

Fig figure

FR framework region

g earth acceleration

GC germinal center

HLA human leukocyte antigen

HSP heat shock protein

Ig [G, M, A] immunoglobulin [class G, M, A]

IgV immunoglobulin variable region

IP immunopercipitation

IPTG Isopropyl-(-D-thiogalactopyranoside)

kD kilo Dalton

LB-Medium Luria Broth-Medium

MHC major histocompatibility complex

M-MLV Moloney Mouse Leukemia Virus

MOPS (3-[N-Morpholino]propan)-sulfonsäure

mRNA messenger RNA

OA osteoarthritis

PBS phosphate buffered saline

PCR polymerase chain reaction

PMSF Phenylmethansulfonylfluoride

R/S ratio replacement to silent mutations ratio

RA rheumatoid arthritis

RF rheumafactor

rpm rotations per minute

SDS-PAGE Sodium-Dodecyl-Sulfate Polyacrylamide gel electrophoresis

SLE systemic lupus erythematosus

Tab. Table

TCR T-cell receptor

TE Tris EDTA-buffer

TEMED N´N´N´N´-Tetramethylethylendiamine

TNF tumor necrosis factor

Tris Tris-(hydroxymethyl)-aminoethan

v/v volume per volume percentage

VH variable region heavy chain

VL variable region light-chain

w/v weight per volume percentage

X-gal 5-Bromo-4-chloro-3-indolyl-phosphate

8. ACKNOWLEDGMENTS

To Prof Dr HK Müller-Hermelink for the opportunity to do my doctorate work at the Institute for Pathology, and for assigning me such an interesting theme as rheumatoid arthritis to work on.

To Prof Dr U Zimmermann for accepting to be my tutor at the Faculty for Biology, and aid in resolving all formalities concerned with the doctorate thesis.

To Dr V Krenn for orientating me in an excellent manner through the secrets of B-lymphocytes in rheumatoid arthritis. And specially for the good hints concerning my future career.

To Dipl. Biol. R Hermann for all the help he gave me concerning experimental problems, also for the availability to discuss my experimental results, and mainly for his friendship.

To Dr EC Müller (Max Delbrück Center, Berlin) for the protein sequencing and above all for her helpfulness and kindfulness. To PD Dr A König (Orthopedic Hospital, Univ. Würzburg) and Prof Dr G König (Baumann Klinik, Stuttgart) for the valuable tissue samples and clinical data of the patients. To Prof Dr H Burkhardt and Dr HG Kraetsch (Med. Klinik III, Univ. Erlangen) for sending the serum samples used in the COMP-epitope study, and valuable help on the clinical interpretation of the results. To PD Dr C Berek and Dipl Biol HJ Kim (DRFZ, Berlin) for revealing me the secrets of IgVH-analysis. To Prof Dr D Heinegård and Prof Dr R Holmdahl (Univ. Lund, Sweden) for their help on understanding a little more about COMP and COMP animal models.

To the group of Prof Dr HP Vollmers (specially to Dipl Agr. Ök. E Wozniak) and to the group of Prof Dr E Serfling (specially Dr S Tchoupillo) for technical assistance.

To C Kohaut for helpful hints on immunohistochemistry. To E Schmitt for the technical support preparing the photographic documentation.

To my „laboratory mates“ Dr A Thalheimer, cand. Med G Ristow, cand. Med A Ruppert, cand. Med M Stockinger, cand Med S. Geis for the excellent working atmosphere.

To my parents, my sister Maria do Rosário and my brothers Gonçalo and Duarte, for allowing me an unconcerned study-time both in Portugal and Germany, and for supporting me in my decisions.

And, last but not least, to my dear K Born for correcting this work and for his love and support.

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Sep 1992 - Sep 1996- Studium der Biochemie an der Wissenschaftlichen Fakultät der Universität Lissabon (Portugal)

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10. Publications List/ Veröffentlichungen

10.1. Original Articles/ Originalarbeiten

1. Souto-Carneiro MM, Krenn V, Hermann R, König A, Müller-Hermelink HK (2000). IgVH Genes from Different Anatomical Regions -with Different Histopathological Patterns- of a Rheumatoid Arthritis Patient Suggest Cyclic Re-entry of Mature Synovial B-cells in the Hypermutation Process. Arthritis Research: in press.
2. Souto-Carneiro MM, Krenn V, Hermann R, Ristow G, König A, Müller-Hermelink HK. (1999). Mutational pattern in the VH-genes from B-cells taken from different anatomical locations of a rheumatoid arthritis patient. Verh Dtsch Ges Path 83: 260-269
3. Krenn V, Souto-Carneiro MM, Kim HJ, Berek C, Starostik P, Müller-Hermelink HK. (July 2000). Histopathology and molecular apthology of synovial B-lymphocytes in rheumatoid arthritis. Histol Histopathol: in press
4. Krenn V, Hensel F, Kim HJ, Souto-Carneiro MM, Ristow G, König A, Vollmers HP, Müller-Hermelink HK. (1999). Molecular IgV_H analysis demonstrates highly somatic mutated B-cells in synovialitis of osteoarthritis: A degenerative disease is associated with a specific, not locally generated immune response. Lab Invest 79(11): 1377-84.
5. Krenn V, König A, Hensel F, Berek C, Souto-Carneiro MM, Haedicke W, Wang YK, Vollmers HP, Müller-Hermelink HK. (1999). Molecular analysis of rheuma factor (RF)-negative B-cell hybridomas from rheumatoid synovial tissue: evidence for an antigen-induced stimulation with selection of high mutated IgVH and low mutated IgVL/λ genes. Clin Exp Immunol 115: 168-175.

10.2. Published Abstracts/ Veröffentlichten Abstracts

1. Souto-Carneiro MM, Krenn V, Müller EC, Otto A, König A, Müller-Hermelink HK. (1999). Novel organ specific human rheumatoid autoantibody (IgG2λ) with specificity for cartilage oligomeric matrix protein (COMP). Immunobiol 200 (3-5): 679.
2. Souto-Carneiro MM, Krenn V, Müller EC, Hermann R, et al. (1999). Human synovial B-cell hybridoma from rheumatoid arthritis with specificity for cartilage oligomeric matrix protein. Arth Rheuma 42(9) (Suppl): 178.
3. Souto-Carneiro MM, Krenn V, Ristow G, König A, Berek C, Müller-Hermelink HK. (1999). Mutational pattern in VH-genes from B-cell taken from different anatomical regions of a rheumatoid arthritis patient. Path Res Pract 195 (5): 317.
4. Souto-Carneiro MM, Krenn V, Hensel F, Hermann R, König A, Berek C, Müller-Hermelink HK. (1998). VH-genes analysis from different anatomical regions from a rheumatoid arthritis patient suggest the existence of an antigen-driven B-cell response. Immunobiology 199: 668.
5. Souto-Carneiro MM, Krenn V, Hermann R, König A, Berek C, Müller-Hermelink HK. (1998). Rheumatoid synovial B-cells show hypermutated VH genes with aminoacids deletions on the complementarity determining regions. Z Rheum 57 Suppl 1: 33.
6. Thalheimer A, Souto-Carneiro MM, Müller-Hermelink HK. (1999) Mutational analysis of VH genes in microdissected germinal centers of a follicular lymphoma suggests an antigenic selection“ Immunobiology 200 (3-5): 617.
7. Ristow G, Krenn V, Souto-Carneiro MM, König A, Kramer C, Müller-Hermelink HK. (1999). Immunophenotyping of the inflammatory infiltrate and expression of interleucin 2 and interleucin 10 in osteoarthrosis. Path Res Pract 195 (5): 317.
8. Gerhard N, König A, Souto-Carneiro MM, Kramer C, Krenn V. (1999). Analysis of IgVH genes from synovial B-lymphocytes from psoriasis arthritis. Path Res Pract 195 (5): 317.

9. Krenn V, Kim HJ, Berek C, Souto-Carneiro MM, Hensel F, Ristow G, König A, Müller-Hermelink HK. (1999). Molecular analysis of IgVH genes from synovial B-cells in arthrosis and rheumatoid arthritis. Path Res Pract 195 (5): 305.
10. Krenn V, Souto-Carneiro MM, Hensel F, König A, Berek C, Müller-Hermelink HK. (1998). B-lymphocytes' maturation takes place in the perivascular region of synovial tissue from osteoarthrosis patients. Immunobiology 199: 660.
11. Krenn V, Hensel F, Berek C, Souto-Carneiro MM, König A, Müller-Hermelink K. (1998), Evidence for B-lymphocytes' maturation on synovial tissue without germinal centers from osteoarthrosis patients. Z Rheum 57 Suppl 1: 65.

10.3. Prizes/ Preise

1. 2nd Poster Prize 83rd Congress of the German Society for Pathology (Jena, May 1999): Souto-Carneiro MM, Krenn V, Ristow G, König A, Berek C, Müller-Hermelink HK. Mutational pattern in VH-genes from B-cell taken from different anatomical regions of an rheumatoid arthritis patient
2. 3rd Poster Prize 31st Congress of the Medical Polyclinic from the Univ. Würzburg (May 1999): Schultz H, Krenn V, Starostik P, Souto-Carneiro MM, Tony HP. Oligoarthritis vermittelt durch Tumor-spezifische T-Lymphocyten als Primärmanifestation eines okkulten Nierenzellkarzinoms.
3. Travel Award from the Interdisziplinäreszentrum für klinische Forschung (IZKF) from the University of Würzburg (April 2000).

10.4. Communications/ Vorträge

1. July 1997, Deutsches Rheumaforschungszentrum (Berlin): "Influence of lpr and gld mutations in the development of CIA on an intermediate resistant mouse strain"

2. March 1998, 14th Spring Congress of the German Society for Immunology (Frankfurt a.M.): “Analysis of IgVH expression in synovial B-cells taken from rheumatoid arthritis and osteoarthrosis patients”
3. June 1998, Rheumakreis der Univ. Würzburg (Würzburg): „IgVH-Gene aus verschiedenen Lokalisationen einer RA Patientin weisen auf einen konservierten Mutationsmuster auf.
4. September 1998, 28th Congress of the German Society for Rheumatology (Baden-Baden): “Rheumatoid synovial B-cells show hypermutated VH genes with aminoaid deletions in the complementarity determining regions”
5. October 1998, European Science Foundation Conference on B-cells in health and disease (Acquafredda de Maratea, Italy): “VH-genes analysis of B-cells from different anatomical regions from a rheumatoid arthritis patient suggests that only a restricted number of antigens is involved in the pathogenesis of RA”
6. November 1998, 25th Congress of the Portuguese Society for Immunology (Porto, Portugal): “VH-genes analysis of B-cells from different anatomical regions from a rheumatoid arthritis patient suggests a conserved antigen-driven mutational pattern”
7. March 1999, Experimental Rheumatology Workshop (Berlin): “Characterisation of antibodies produced by RA synovial B-cell hybridomas”
8. October 1999, 30th Annual Congress of the German Society for Immunology (Hannover): „Novell organ-specific human rheumatoid synovial B-cell hybridoma with specificity for COMP“
9. November 1999, 63rd Annual Meeting of the American College for Rheumatology (Boston, USA): „Human synovial B-cell hybridoma from rheumatoid arthritis with specificty for cartilage oligomeric matrix protein“

11. EIDESSTATTLICHEN ERKLÄRUNGEN

Hiermit erkläre ich ehrenwortlich, daß die vorliegende Dissertation "*Molecular and functional analyses of human synovial B-lymphocytes in rheumatoid arthritis*" selbständig am Institut für Pathology der Universität Würzburg angefertigt wurde, und daß keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Würzburg, 30. Mai 2000

(Dipl Biochem Maria Margarida Souto-Carneiro)

Hiermit erkläre ich ehrenwortlich, daß die vorliegende Dissertation "*Molecular and functional analyses of human synovial B-lymphocytes in rheumatoid arthritis*" in gleicher oder ähnlicher Form noch nicht in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 30. Mai 2000

(Dipl Biochem Maria Margarida Souto-Carneiro)

Hiermit erkläre ich ehrenwortlich, daß ich bisher noch keine akademische Grade erworben oder zu erwerben versucht habe.

Würzburg, 30. Mai 2000

(Dipl Biochem Maria Margarida Souto-Carneiro)