Invited Review

Histopathology and molecular pathology of synovial B-lymphocytes in rheumatoid arthritis

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Summary. 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.

Key words: B-cells, rheumatoid arthritis, arthritogenic antigens

Abreviations

RA: rheumatoid arthritis; HSP: heat shock proteins; CDR: complementarity determining region; FR: framework region; IgVH: immunoglobulin heavy chain variable region; IgVL: immunoglobulin light chain variable region; COMP: cartilage oligomeric matrix protein; SLE: systemic lupus erithematosus; FDC: follicular dendritic reticulum cell.

Introduction

Intrasynovial B-cells (including B-lymphocytes and plasma cells) are a constant and dominating component (Fig. 1a,d) of the inflammatory infiltrate in rheumatoid arthritis (RA) (Randen et al., 1995; Krenn et al., 1996a; Stiehl, 1997). Histopathologically, the synovial B-cells may exhibit a follicular distribution (Fig. 1a,b) and may be located in the area of bone cartilage destruction (Fig. 1e), and finally there is a correlation between the serum level of rheuma factors (RF) and the follicular organisation of the synovial B-cells (Randen et al., 1995). These findings indicate a pathogenic role of synovial B-cells in RA. Analysis of the pathogenetic potential of synovial B-cells in RA leads to two important questions: 1) Does an antigen-activated B-cell maturation occur in the synovial tissue? 2) Against which antigens are these B-cells directed?

The first question should provide clarification, whether the local accumulation of B-cells is antigen-dependent or antigen-independent. The second question may lead to the identification of antigens which are

involved in chronic synovitis (arthritogenic antigens).

The organisation of synovial B-cells

The inflammatory infiltrate of chronic RA synovialitis is basically constituted by the functional elements of a secondary lymphatic tissue, including T- and B-cells, 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-cell maturation. In specialized structures of the lymphatic organs (Fi. 2a), the so called germinal centers (GC) of the secondary follicle, antigen-activated B-cell affinity maturation takes place (Berek et al., 1991): unactivated B-cells (naive B-cells) and a subpopulation of CD4-positive T-lymphocytes (Miller et al., 1995) 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-cells. This cellular interaction leads to cell proliferation and immunoglobulin gene hypermutation (IgVH and IgVL genes), which modifies the affinity repertoire of the B-cells (affinity maturation) (Berek et al., 1991; Krenn et al., 1999). Finally, the B-cells 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 past-GC B-cells represent antigen-activated B-cells with a specific set of B-cell-receptors with either low or highly diversified VH-genes (Berek et al., 1991; Camacho et al., 1998). This process occurs in the secondary lymphatic organ B-cells, where high-
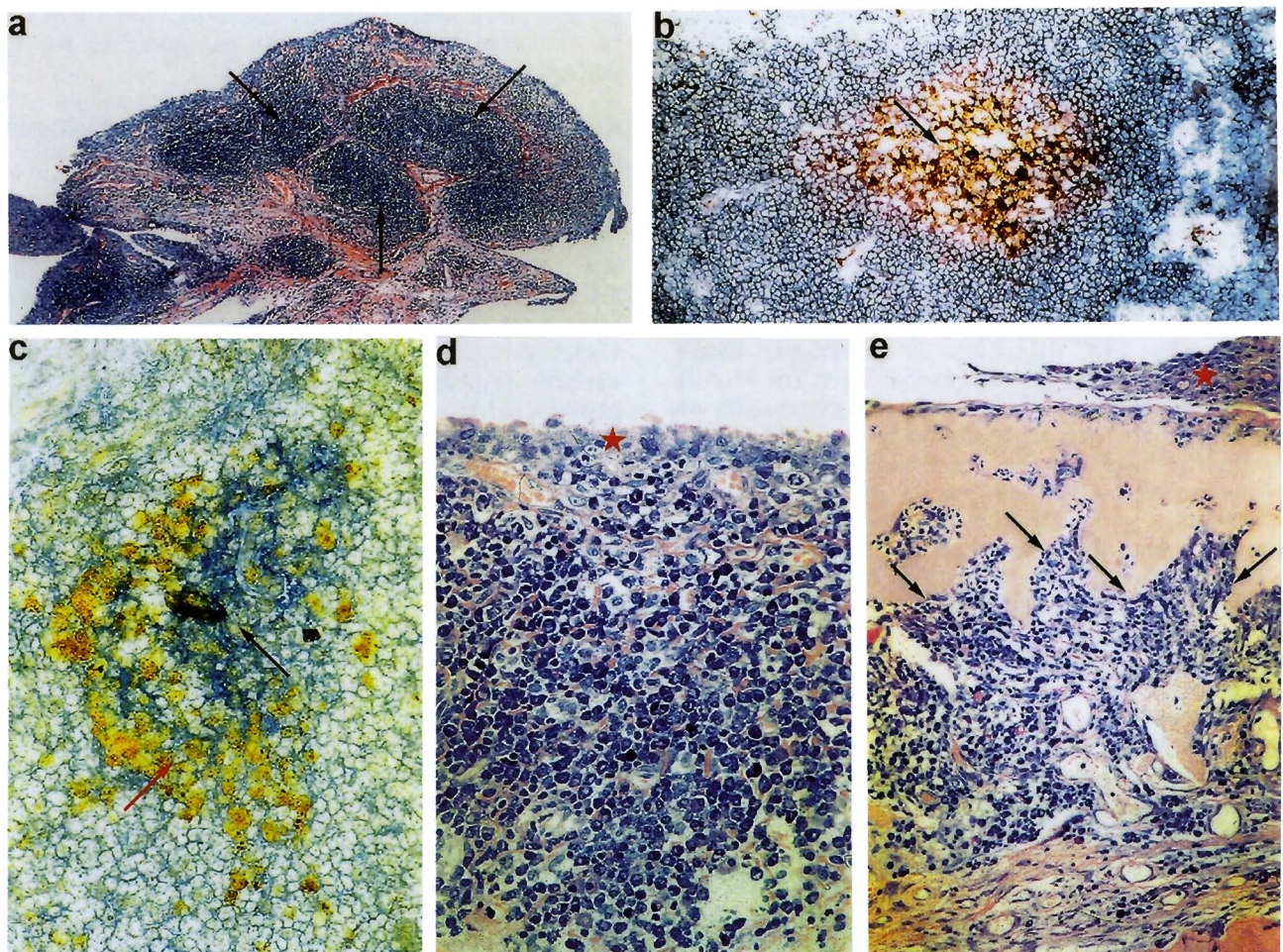

Fig. 1. Histopathology and histology of rheumatoid synovial tissue. **A.** Rheumatoid synovial tissue with villous hypertrophy and lymphofollicular (arrows) inflammatory infiltration (type I, according to Stiehl (1997)). **B.** Double staining immunohistochemistry, demonstrating a lymphatic follicle with centrally located FDCs (Ki-M4+; 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 small 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). A, x 50; B and E, x 100; C and D, x 250

affinity (post-GC B-cells) are produced under the control of FDCs, which represent the antigen-induced and T-cell-dependent immune response (MacLennan, 1994; Camacho et al., 1998).

Compared to the secondary lymphatic organs, the RA synovial tissue often reveals a cellular composition reminiscent of that seen in the lymphatic tissue of secondary lymphoid organs (Randen et al., 1995; Krenn et al., 1996b; Wagner et al., 1998). The pattern of FDC distribution may be immunohistochemically classified (Krenn et al., 1996b): 1) GC pattern (Fig. 2b); 2) GC-like pattern (also called "dysmorphic follicle") (Fig. 2c); and 3) Diffuse distribution pattern of single FDCs (Fig. 2d). Rheumatoid synovial tissue with immunohisto-chemically 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 lymph nodes (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 (Krenn et al., 1996b).

The existence of secondary follicles and follicle-like structures in the synovial tissue indicates that an intrasynovial antigen-driven maturation of B-cells may take place. Using a single cell isolation method, recent studies (Schröder et al., 1996; Kim et al., 1999) have proven experimentally that a local maturation of non-mutated B-cells into highly mutated plasma cells occurs in the synovial tissue of RA. B- and plasma cells did not carry identical rearrangements, but were clonally related, indicating that B-cells underwent a terminal differentiation in the synovial tissue (Fig. 3).

These somatically hypermutated plasma cells may even present amino acids deletions in their VH-genes, which have been described in secondary lymphatic
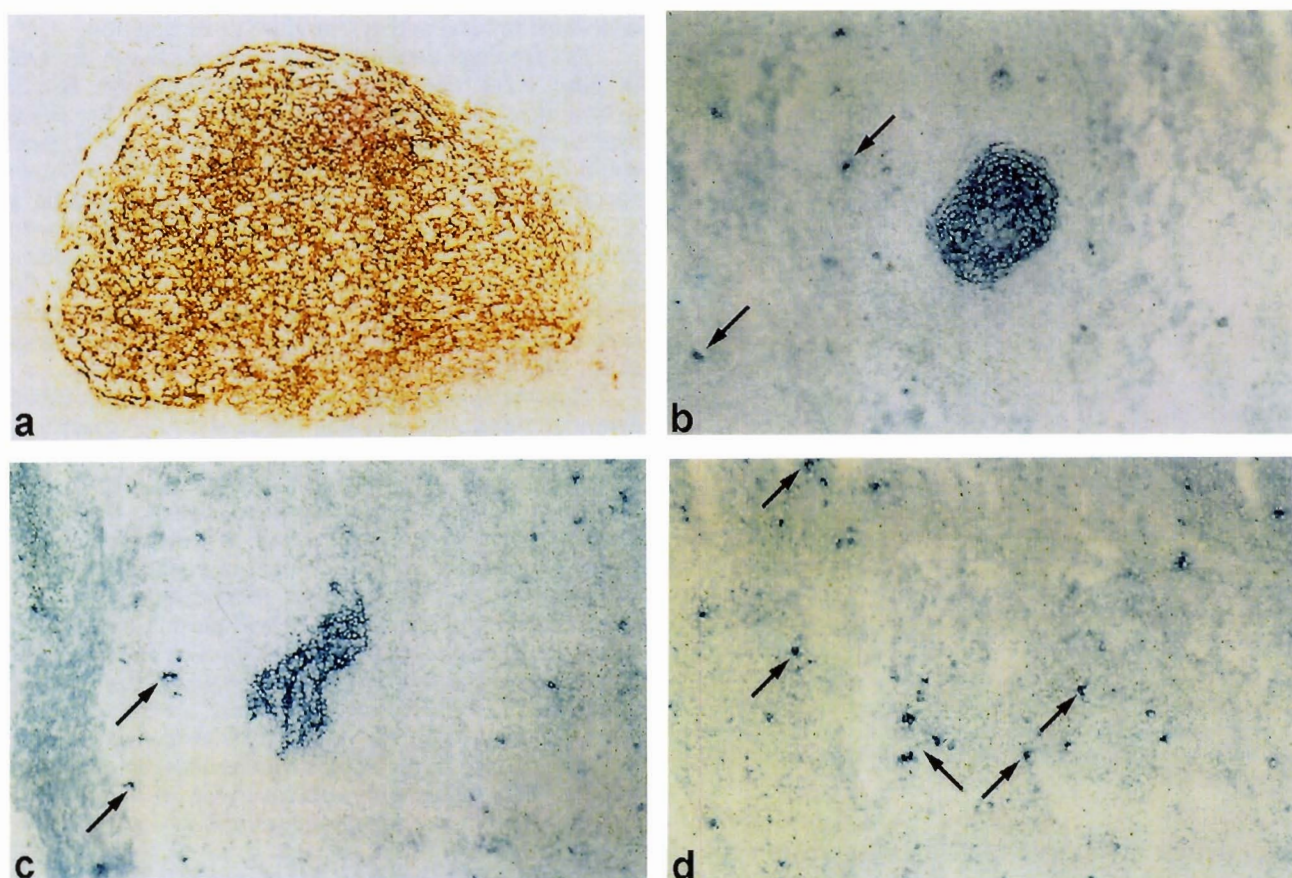

Fig. 2. Patterns of FDC distribution in: **(A)** normal lymphatic tissue (tonsil) and rheumatoid synovial tissue exhibiting a germinal center pattern; **(B)** a germinal center-like pattern and **(C)** a diffuse distribution of single FDCs **(D)**. Arrows point at single FDCs. x 100
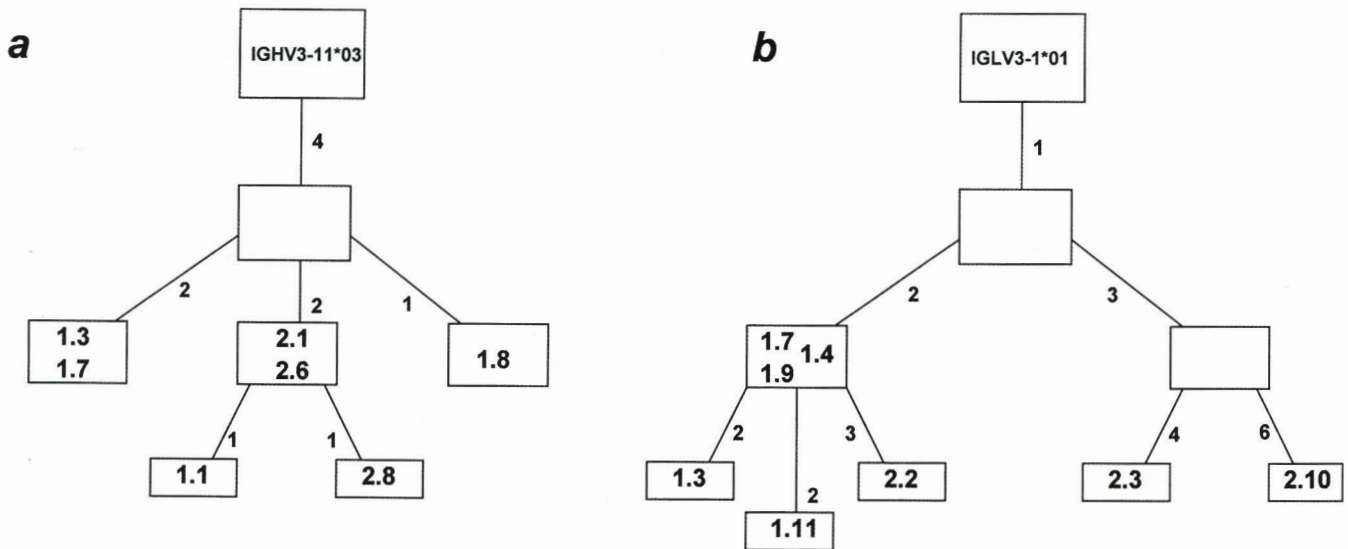
a

b

Fig. 3. 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 Kim et al. (1999).

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            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
Syn 194/1   --- --- --- --- --- --- --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
Syn 194/1   -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
Syn 194/1   --- --- --- --- --- --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
Syn 194/1   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
Syn 194/1   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
Syn 194/1   -T- --- --- --- --- --- --- --- C-- --- --- ---
            F                                   P

            A   V   Y   Y   C   A   R
IgHV1-18*01 GCC GTG TAT TAC TGT GCG AGA
Syn 194/1   --- --T --C --T --- A-- --G
                                T
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Fig. 4. Comparison of IgVH sequence Syn 194/1 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.

organs (Wilson et al., 1998). The existence of such deletions in RA synovialitis (Fig. 4) (Souto-Carneiro et al., 2000), therefore, stresses the functional homology to the secondary lymphatic tissue. On the other hand, they can also be seen as a way of generating new specificities, such as, for example, autoreactive antibodies that could contribute to local and systemic tissue destruction.

The findings obtained from synovial tissue are well in line with the findings obtained from B-cell hybridomas. Depending on the specificity of the B-cell hybridomas, unmutated and highly mutated IgVH genes could be detected. In two of these hybridomas of known specificity (one to a mitochondrial antibody (Krenn et al., 1998), and the other to cartilage oligomeric matrix protein (COMP) (Souto-Carneiro et al., 1999) produced in our laboratory) the values of the R/S ratios in the CDR were higher than 2.9, which indicates that these B-cells belong to the pool of post-GC B-cells (Shlomchick et al., 1987; Chang et al., 1994; Krenn et al., 1999).

The analysis of synovial B-cell clones from blood, synovial fluid, and synovial tissue with RF-specificity (immunoglobulins with specificity for the Fc portion of immunoglobulins) shows partially unmutated IgVL-gene sections, so that RF-producing B-cells belong both to the classes of post-GC and naive B-cells. 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- 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-cell specificities could, therefore, help to identify antigens which are involved in local B-cell expansion and immunopathogenesis of RA.

The repertoire of synovial B-cell specificities:

The specificity spectrum of B-cells described in the

literature is wide and ranges from serum antibodies to experimentally produced B-cell clones from peripheral blood, bone marrow, synovial fluid, and synovial tissue (Burastero et al., 1990; Ezaki et al., 1991; Lu et al., 1993; Randen et al., 1993; Borretzen et al., 1997; Krenn et al., 1999). The following specificities have been defined: specificity from hybridomas; RF (Randen et al., 1993); ANCAS and ANAS (Mulder et al., 1993); collagen type II (Emmrich et al., 1995, Rudolphi et al., 1997); HLA-DR (Marnell et al., 1990); thyreoglobulin, tetanusoxoid, DNA, and actin (Randen et al., 1989); human (Krenn et al., 1996a) and bacterial HSP (Krenn et al., 1996a; Rudolphi et al., 1997), filaggrin (Schelekens, 1998). These specificities can be divided into two specificity groups: antibodies with specificity against "self antigens", and antibodies with specificity against "non-self antigens".

Synovial B-cells 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 (ex: antibodies against myocardial components after myocardial infarct). Most of these autoantibodies possess a physiological function in binding antigens which are harmful to the organism (so-called antigen clearance). The existence of autoantibodies in immune disease is, consequently, not necessarily connected to a pathogenic role of the antibody. Many antibodies possess, due to their direct association with diseases, 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-Rose-Koch criteria (Shoenfeld, 1994): 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 heretofore described, these criteria have not been totally fulfilled, mainly due to the lack of a suitable animal model for RA.

Polyreactive antibodies from synovial B-cells:

Antibodies with specificity for several different antigens are described as polyreactive antibodies. Polyreactive antibodies are mainly produced by CD5-positive B-cells and fulfill an important function in the primary immune phase against bacteria and viruses through their "polyreactivity". Interestingly enough, in the synovial membrane the number of CD5+ B-cells is quite elevated (Hassan et al., 1996). How is an elevated number of B-cells, producing polyreactive antibodies, associated with a pathogenic function? Since CD5+ B-cells are regarded as naive B-cells with germline configuration of their IgVH genes, the participation of CD5+ B-cells in synovialitis could reflect the genomic dependence of this disease (association of RA to certain groups).

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

Monoreactive antibodies from synovial B-cells:

Rheumafactors

These were the first autoantibodies described in RA (Waller, 1940). 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-cells show high affinity for IgG3 (Randen et al., 1995). An immunohistochemical analysis showed that about 70% of the synovial IgM-producing B-cells, 50% of the IgG-producing B-cells, and 20% of the IgA-producing B-cells are RF-producing cells (Youinou et al., 1984). 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 (Zvaifler, 1973).

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. (2000) 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 Gc 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 mutatin that does not affect the "classical" antigen binding sites. Furthermore, they suggest that the RF receptor on B-cells binds both antigen and IgG Gc 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 (Olee et al., 1992), 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 IgF enhanced by the presence of antigens (Sutton et al., 2000).

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-cells for the pathogenesis of cartilage destruction: at first, destruction may be induced by the passive transfer of collagen type II-specific antibodies (Terato et al., 1992); additionally, this disease may be induced through the immunisation with triplehelical collagen, which is exclusively recognized by B-cells, but not by T-cells (Burkhardt et al., 1992). Only B-cells 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-cell clone produced by electrofusion showed -according to indirect immunofluorescence technique- a mitochondrial pattern in the stomach mucosa, which was then confirmed by immunoelectron microscopy (Krenn et al., 1998). Western blot analysis of a mitochondrial preparation showed a 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 =2, indicating that the B-cell 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.

Synovial B-cells 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 (De Graeff-Meeder et al., 1991). The remarkable conservation of amino acid sequences between bacterial and human HSP (Jones et al., 1993) 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-cells as well as B-cells from rheumatoid synovial fluid and tissue were shown to be specific for bacterial HSPs and human (De Graeff-Meeder et al., 1990; Krenn et al., 1996a; Rudolphi et al., 1997). Since HSPs are expressed in the synovial tissue, a humoral HSP60 response initially directed against an infectious agent could cause synovialitis by crossreactivity. Here again, the question is why the crossreactivity is restricted to synovial tissue, since HSPs show a ubiquitous expression.

A recent study, performed by Kowal et al. (1999) 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 may 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.

Conclusions and future directions

The relatively high number of B-cells, and the local antigen-dependent expansion of B-cells in synovial tissue, indicate an important function of synovial B-cells in the pathogenesis of RA. The pathogenic relevance of B-cells in RA is supported by the transgenic KRNxNOD mouse model (Kouskoff et al., 1996), and by the exclusive capacity of B-cells to recognise conformation-dependent antigens, which are arthritogenic in animal models (type II collagen). The demonstration of secondary lymphatic follicles and follicle-like structures containing FDCs (dysmorphic follicles) and the local B-cell expansion accompanied by differentiation into plasma cells points towards an antigen-induced process in the RA-typical tissue lesion. Synovial B-cells from RA patients exhibit specificity for the Fc region of IgG (RF), type II collagen, DNA, COMP, actin, bacterial HSP, and mitochondrial antigen. Antibodies that simultaneously show specificity for self and non-self antigens (ex: anti-HSP antibodies) may, due to crossreaction, represent a connection between "non-self" and "self" antigens, and help with the characterisation of exogenous arthritogenic antigens. The further characterisation of rheumatoid B-cell hybridomas, and the experimental expression of IgVH and IgVL genes isolated from rheumatoid synovial tissue will help to characterise the antigens involved in chronic synovialitis and pathogenesis of RA.

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