



**P3**

**Clinical sensitivity of antibodies against cyclic citrullinated peptide in patients with rheumatoid arthritis**

**B Božič, S Čučnik, A Ambrožič, B Lestan, M Kos-Golja, B Rozman B and T Kveder T**

*University Medical Centre, Department of Rheumatology, Ljubljana, Slovenia*

The synthetic cyclic citrullinated peptide (CCP) is recognised by rheumatoid arthritis (RA) associated antifilaggrin antibodies, previously determined as antikeratin antibodies or perinuclear factor. Antibodies detected by ELISA using CCP as an antigen (anti-CCP) seem to be of prognostic value in patients with RA.

The objective of our study was to determine the clinical sensitivity of anti-CCP in patients with definite RA (according to ARA diagnostic criteria). RF results were considered when measured in the same serum as anti-CCP.

Sera from 97 RA patients (15 M, 82 F) were tested for anti-CCP in duplicates by Immunscan RA ELISA (Euro-diagnostica). RF was tested in the same serum in 69/97 patients.

In all 4 assay runs, OD of all calibrators vs. their calculated values completely corresponded to the figure in the manufacturer's analysis certificate. When the units of the lowest calibrator D were calculated by the equation of log calibration curve according to the manufacturer's protocol, they were always about 20% (7-17U) above the defined value (50U). This inconsistency of the curve fitting led to a wrong validation in 26/97 (27%) of the RA samples which OD below the OD of the calibrator D, but with calculated results above the defined cut-off at 50U. Therefore, we calculated results by the equation of 3rd degree polynomial curve which fitted perfectly the measured OD values to the defined units.

Out of 97 RA patients, 56 were anti-CCP positive (58%). From 69 patients simultaneously tested for RF and anti-CCP, both tests were positive in 24/69 (35%) and both negative in 23/69 (33%). Anti-CCP were positive in 15/38 (40%) patients with negative RF. Despite lower anti-CCP positivity rate, 16% of samples in RF neg. group exhibited high anti-CCP values:

Anti-CCP (U)	<50 (neg)	50–200	201–800	801–3200	>3200
RF pos (n = 31)	7 (23%)	4 (13%)	6 (19%)	5 (16%)	9 (29%)
RF neg (n = 38)	23 (60%)	6 (16%)	3 (8%)	4 (11%)	2 (5%)

The clinical sensitivity of the anti-CCP test in our RA patients was 58%, which is lower than previously reported (68%). The discrepancy might partially be due to different calculation of the results. We believe that the introduction of polynomial standard curve could contribute to a more consistent validation of samples exhibiting OD below the lowest calibrator.

Our results support the idea that anti-CCP are of diagnostic value especially in RF neg patients.

**P4**

**Expression of citrullin-containing antigens in RA synovium**

**TJ Smeets, ER Vossenaar, MC Kraan, WAM van Mansum, JM Raats, WJ van Venrooij, PP Tak**

*Academic Medical Center, Amsterdam and University of Nijmegen, Nijmegen, The Netherlands*

**Introduction:** The presence of autoantibodies directed to citrullinated antigens in serum is highly specific for RA. The aim of this study was to compare anti-CCP concentrations in paired serum/SF

samples of patients with RA and to investigate whether this is associated with the expression of citrullinated antigens in RA synovium and to study the nature of these antigens.

**Methods:** A recombinant single-chain variable fragment (scFv) monoclonal antibody was selected against a cyclic citrullinated peptide (CCP) from a patient antibody-fragment phage-display library. This scFv and patient antibodies affinity purified with CCP, were both used for immunohistochemical staining of synovial cryostat sections of RA (30) and control patients (OA (13), ReA (9), and other arthritides (28)). In addition, rabbit anti-citrullin antibodies (Biogenesis) were used for immunohistochemistry of synovial cryostat sections of RA (14), and control patients (OA (10), ReA (7), and other arthritides (23)). IgG anti CCP titers were calculated with the quantitative Rapsan RA ELISA kit (Eurodiagnostica). Total IgG concentrations were determined on a cobas Fara-2 centrifugal analyzer.

**Results:** Citrullin containing antigens were observed in synovial cryostat sections of anti-CCP positive and negative patients. Staining with ScFv monoclonal antibody was noted in synovial lining cells and in (peri)vascular areas in 13/30 RA patients, 7/13 OA patients, 5/9 ReA patients, and 12/28 other arthritis patients. CCP positivity was on average similar in all diagnostic groups. Staining was absent in the negative controls using a control scFv antibody. Staining with rabbit anti-citrullin polyclonal antibody was noted in 8/14 RA patients, 3/10 OA patients, 2/7 ReA patients, and 6/23 other arthritides. However, controls using irrelevant rabbit antibodies were also positive in some patients in all groups. Anti-CCP concentrations (expressed in Units per mg total IgG) were on average 1.34 times higher in SF compared to serum (n = 20, P < 0.05) or 1.37 when only positive samples were included (n = 11, P < 0.05)

**Conclusion:** Citrullinated antigens are present in the synovia of both RA and control patients with similar prevalence. The presence of anti-CCP autoantibodies in serum is not associated with the expression of citrullinated antigens in the synovium. The identity of the citrullinated antigens and potential differences between RA and control synovia remain to be identified.

**P5**

**Auto-reactivity patterns in rheumatoid arthritis**

**S Behrens\*, F Schumann\*, S Adelt\*, H Hofseß\*, R Bergholz, GR Burmester\*, JM Engel† and S Bläß\***

*\*Department of Rheumatology & Clinical Immunology, Charité University Hospital, Berlin, Germany; †Clinic for Rheumatology, Bad Liebenwerda, Germany*

Rheumatoid arthritis (RA) is characterized by the occurrence of autoreactive antibodies and T cells. RA is heterogeneous disease also with respect to these autoreactivities, since none of them is present in every RA patient and they are additionally also present – although to a considerably lesser extent - in other autoimmune diseases and even in healthy individuals. It has now been analyzed if there are clusters of autoreactivities that are absolutely specific for RA.

Therefore, the RA-associated autoantigens RA33 (hnRNP A2), citrulline, rheumatoid factor (RF), the stress protein BiP (heavy chain binding protein), calpastatin (Calp) and calreticulin (Calr) have either been biochemically purified or used as a kit of recombinant antigen or chemically synthesized peptides. These antigens have been applied to screen sera and PBMCs from RA and control patients for reactivity and the data have subsequently been subjected to cluster analyses.

Analyzing the reactivities of 100 RA and 100 control patients, the following patterns of the three combined autoreactivities were determined to be absolutely specific for RA: RF+Cit+BiP+, RF-Cit+BiP+. RA-specific patterns composed of four autoreactivities are RA33+RF+Cit+BiP+, RA33-RF+Cit+BiP+, RA33+RF+Cit+BiP-, RA33+RF-Cit+BiP+, RA33+RF+Cit-BiP+, RA33-RF+Cit-BiP+.



## P8

### Investigation of the epitopes of human profilaggrin derived peptide targeted by antibodies of patient with rheumatoid arthritis

M Brozik, J Szakonyi<sup>†</sup>, A Magyar<sup>‡</sup>, R. Tóbi<sup>‡</sup>, B Rojkovich<sup>‡</sup>, F Hudecz<sup>‡</sup> and P Gergely<sup>†</sup>

National Institute of Rheumatology, <sup>†</sup>Central Laboratory of Immunology Faculty of Medicine Semmelweis University, <sup>‡</sup>Peptide Chemistry Research Group, Eötvös Lóránd University, Hungarian Academy of Science, Budapest, Hungary

Anti-filaggrin antibodies (AFA), directed against the 37-40 kD epidermal protein filaggrin are one of the most specific markers of rheumatoid arthritis (RA) and include anti-keratin antibodies (AKA) and anti-perinuclear factor (APF). Although the antigen triggering autoimmune response to filaggrin related proteins has not been identified, recent studies confirmed that citrulline is essential constituent of protein related antigenic determinants recognised by RA specific autoantibody population.

The aim of our study was to identify epitopes of filaggrin derived-peptides targeted by RA specific antibodies to provide further information about the nature of the initial autoantigenic substance.

Citrullin containing peptides of human profilaggrin region (amino acid residues 306-324) derived from known cDNA and on the basis of the data published by Shellekens were synthesised by the multipin peptide synthesis on solid support and were reacted in situ by patient sera. Two 19-mer peptides were prepared with single citrullin substitution at position 312 or 321 respectively and four additional ones with simultaneous replacements of two Arg by Cit. Shortened versions of the <sup>306</sup>SHQESTCitGRSGRSGRSGR<sup>324</sup> peptide were also produced by removal of 1-6 amino acid residues from its N-terminal and the 14-mer truncated one was further shortened from its C-terminal as well. The reactivities of these peptides with RA sera and healthy controls were determined. The results showed that substitution of arginine 312 by citrulline plays major role in the antigenicity of filaggrin-derived sequences. Peptides not containing Cit in position 312 almost lost their ability to bind antibodies from RA sera. Replacement of one additional Arg by Cit in different positions did not improve the antigenicity. When the peptide with Cit in position 312 were shortened from its N-terminal, the 14-mer one showed the highest reactivity. Further shortening of this sequence from its C-terminal showed that TXGRS motif seems to be essential to comprise the autoantigenic epitope.

In conclusion our results provide further evidence that not simply the presence of citrullin but also the nature of its surrounding amino acids have important role in the creation of autoantigenic epitope reactive with anti-filaggrin antibodies.

## P9

### Anti-skin anti-intercellular antibodies in juvenile idiopathic arthritis

I Hromadnikova, P Vavrincova, K Stechova, D Hridelova and J Vavrínek

2nd Paediatric Clinic, University Hospital Motol, Prague, Czech Republic

The aim of this work was to study the presence of anti-skin anti-intercellular (ASA-IC) and anti-basement membrane (ASA-BM) antibodies of the IgG class in patients with juvenile idiopathic arthritis (JIA) without clinical features of chronic vesicular-bullous diseases including pemphigus, pemphigoid and epidermolysis bullosa acquisita (EBA).

No D-penicillamine was used for JIA management in this group due to a risk of drug-induced pemphigus. Indirect immunofluorescence antibody test (IIF) and dual substrates of monkey and guinea pig esophagus sections were used for the detection and quantification of ASA-IC as well as ASA-BM antibodies. Overall ASA-IC were detected in 50 out of 57 studied patients' sera samples (87,7 %,  $P = 0,0003$ ) ranging from 1:20 to  $\leq 1:320$  dilutions. Respective of the classification criteria for idiopathic arthritis of childhood ASA-IC were observed in 6/6 patients with systemic disease (100%,  $P = 0,029$ ), 24/29 patients with RF negative polyarthritis (82,7 %,  $P = 0,01$ ), 16/18 RF positive polyarthritis (88,9 %,  $P = 0,0077$ ) as well as in a small cohort of patients with oligoarthritis (2/2) and psoriatic arthritis (2/2). However we have observed a high incidence of anti-skin anti-intercellular antibodies in a cohort of patients with JIA we suggest that subclinical pemphigus occurring in this group might be exacerbated with different stimulus including pemphigus inducing drugs. No ASA-BM antibody positivity was observed in a cohort of 57 studied patients.

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## P10

### Correlation of the humoral immune answer to selected bacterial antigens with presence of the DNA specific to *Salmonella enteritidis* after amplification by PCR

J Zabek, J Noworyta, M Kurowska, M Brasse-Rumin, J Gago, B Kwiatkowska and H Garwolińska

Department of Microbiology and Serology, Institute of Rheumatology, Warsaw, Poland

**Introduction:** An infectious aetiology has often been discussed as a most compatible with both the clinical and pathological features of rheumatoid arthritis (RA). Until now, no single microorganism can be shown as consistently associated with development of RA. In our former serological and molecular studies we have shown that the most common humoral immune answer in RA patients is directed to *Salmonella enteritidis* /S. ent./ antigens, especially to specific for *Salmonella enteritidis* O3 LPS.

The aim of the study was to prove the correlation between systemic and local humoral immune answer to *Salmonella enteritidis* antigens and the presence of DNA specific for *Salmonella enteritidis* O3 serotype.

**Materials and methods:** In the tested group, composed of 35 sera and 20 synovial fluid, taken from 20 patients with connective tissue diseases the presence of DNA after PCR amplification and antibodies by ELISA method were estimated.

**Results:** In 10 of 35 (31%) synovial fluids /bacteriologically negative/ we have found /after amplification by PCR/ double band of the DNA, specific for *Salmonella enteritidis*, possessing mol. weight 390bp and 420bp respectively. Also in the same group of patients the antibodies to OMP S. ent. in 30% of tested cases, to LPS S. ent. in 78,9%, in 30% to ECA and none to peptidoglycan have been found. Only in a few of the PCR-positive synovial fluid elevated level of antibodies to S. ent. have been found.

**Conclusions:** No evident correlation, so far, between class and specificity of humoral antibodies and the presence of specific for S. ent. DNA after PCR amplification have been found.



overcame the suppressive effect and allowed an increased proliferation. This argues strongly for the presence of BiP-specific regulatory T cells restricted for HLA-DR and BiP-specific effector T cells restricted for HLA-DP and -DQ in this subset of RA patients. These effects could not be mimicked by blocking anti-IL-10 or anti-TGF- $\beta$  antibodies, implicating that other factors or also direct cell-cell contact are required.

Apparently, the healthy immune system views BiP as a component to which autoreactivity is either avoided or tightly regulated. In RA this general principle appears to have lost control. BiP-reactive may serve as a new diagnostic marker in RA, while regulatory T cells may provide means for a specific therapy.

#### P14

### Lysozyme and its biological value in rheumatoid arthritis (RA)

J Smirnow and M Wislowska

Central Clinical Hospital, 137 Woloska Street, Warsaw, Poland

Lysozyme or muramidase catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan. A basic enzyme that is present in saliva, tears, egg white and many animal fluids. Its function is an antibacterial agent. Lysozyme is well known for the ability to hydrolyze the cell wall of bacteria.

**Objective:** The aim of study was to measure the concentration of lysozyme in synovial fluid in RA patients.

**Methods:** We measured the lytic activity of lysozyme towards *micrococcus lysodeikticus* (1, 2, 3), bacteria which are highly susceptible to lysis by lysozyme by the turbidometric method 30 synovial fluid of RA patients. In order to obtain a method covering a wider range of lysozyme concentrations, Osserman and Lawlor worked out the so-called lyso-plate method (4).

The test measured the zone of clearing by lysozyme in an agar plate, in which *micrococcus lysodeikticus* is embedded. After about 18 hours the diameter of the zone of clearing is measured.

**Results:** In all our RA synovial fluid we observed increased level of lysozyme.

**Conclusions:** The increased levels of lysozyme in synovial fluid in RA could indicate of monocyte/macrophage activity and might be used to study disease activity in RA.

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## Poster Discussion B

### Cytokines

#### P15

### Differential effect of corticosteroid therapy on the cytoplasmic cytokine expression in CD4 and CD8 positive T cells from lupus patients

E Kiss, M Aleksza, P Antal-Szalmás, S Sipka and Gy Szegedi

3<sup>rd</sup> Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, Hungary

Due to their different antiinflammatory and immunomodulatory effects corticosteroids are widely used in the treatment of SLE. Literature data support both Th1 and Th2 dominance in lupus. There are only few reports about cytokine profile of CD8+ T cells in SLE.

In the present study we examined by flow cytometry the cytoplasmic IFN $\gamma$ , IL-4 and IL-10 expression in CD4+ and CD8+ T cells from six active, untreated, newly diagnosed SLE patients, who received after that 1 mg/kg methylprednisolon. Pretreatment expression of IFN $\gamma$  was lower, while IL-4 and IL-10 expressions were higher in CD4+, but not in CD8+ T cells of patients than in control cells. Corticosteroid treatment increased IFN $\gamma$  and decreased IL-4 and IL-10 expressions in patients' CD4+ cells, but had no significant effect on the cytoplasmic cytokine expression of CD8+ cells.

In conclusion, present results indicate that corticosteroid therapy does not influence IFN $\gamma$ , IL-4 and IL-10 expression in CD8+ T cells of lupus patients, and it may have pathogenic significance.

#### P16

### Elevated IL-16 level is a non-genetic characteristic of patients with severe systemic lupus erythematosus

LR Lard, BO Roep\* and TWJ Huizinga

Departments of Rheumatology and Immunohaematology and Bloodbank\*, Leiden University Medical Center, Leiden, The Netherlands

**Introduction:** IL-16, originally named lymphocyte chemoattractant factor, is a cytokine which is mainly produced by CD8+ T cells. Several reports have described that increased levels of IL-16 are in part responsible for T cell abnormalities in SLE patients. It is unknown if the previously reported increased levels of IL-16 is a characteristic underlying susceptibility to SLE or is a characteristic of the disease itself.

**Methods:** Accumulated organ damage was measured with the SLICC/ACR Damage Index. Twenty-five severe (SLICC/ACR: 4.9  $\pm$  2.5) and ten non-severe (SLICC/ACR: 1.0  $\pm$  0.8) SLE patients were included in this study. Also 11 first degree relatives and 12 healthy volunteers were included in this study. Plasma IL-16 levels were measured by ELISA.

**Results:** No significant difference in the IL-16 levels of the first degree relatives of patients with SLE (38.3  $\pm$  11.1 pg/ml) were observed when compared to controls (31.2  $\pm$  10.1 pg/ml). In order to analyze characteristics of the SLE in relation to concentration of IL-16, IL-16 was measured in severe SLE patients (71.3  $\pm$  87.4 pg/ml;  $P = 0.025$ ) compared to healthy controls. On the other hand, no significant differences were observed between the non-severe SLE patients (37.8  $\pm$  26.1 pg/ml) and controls.

**Conclusion:** No evidence for increased IL-16 levels in first degree relatives of the SLE patients was observed. IL-16 is enhanced in SLE patients with a severe disease, but not in patients with non-severe disease, thereby suggesting that IL-16 is associated with disease severity, and not with susceptibility for SLE.



1 µg/ml. This could point to a mechanism of action different from the classical neurokinin receptor of SP and CLPs on MO regarding cytokine secretion.

## P20

### Human neutrophil production and cleavage of IL-18: potentiating inflammatory arthritis?

SE Robertson, J Young, FY Liew, IB McInnes and JA Gracie

CRD/Department of Medicine, and Department of Immunology, Glasgow Royal Infirmary, Glasgow, G31 2ER, Scotland

We have recently demonstrated the presence and involvement of IL-18 in rheumatoid arthritis (RA) synovitis. Moreover, blockade of IL-18 *in vivo* is protective in arthritis models. We sought to demonstrate for the first time the production and intracellular processing of IL-18 by human neutrophils. Thereafter we investigated novel processing pathways and potential regulatory mechanisms for IL-18 bioactivity that could operate in synovium.

**Methods:** Matched peripheral blood (PB) and synovial fluid (SF) neutrophils were isolated by ficoll density gradients. IL-18 was detected in neutrophil total protein lysates by western blotting. Serum free neutrophil culture supernatants were incubated with recombinant IL-18 prior to HPLC fractionation and assessed for biological activity using IL-18 sensitive KG-1 cells.

**Results:** Western blotting of neutrophil lysates isolated from PB and SF of rheumatoid and psoriatic arthritis patients demonstrated the presence of a number of IL-18 specific bands ranging in molecular weight from 22 to 6 kD in size, representing pro, mature and possible IL-18 cleavage products. HPLC purified culture supernatants from PB and SF neutrophils contain heat sensitive enzymatic activity capable of *in vitro* cleavage of both recombinant pro and mature IL-18. This caspase independent cleavage of IL-18 resulted in the generation of biologically active fragments capable of modulating IL-18 induced IFN- $\gamma$  production by KG-1 cells.

**Conclusions:** These data demonstrate for the first time that modified fractions of IL-18 may be biologically active, suggesting the existence of a novel regulatory mechanism in the IL-1 cytokine family. In light of their rapid accumulation in large numbers within RA joints, our data further suggest that both neutrophils and IL-18 play important roles in disease pathogenesis.

## P21

### Intracellular expression of CXCR3 on rheumatoid arthritis synovial tissue cells

O Krystufkova, J Vencovsky, S Ruzickova, J Sinkora\*, J Niederlova, CA Power†, C Plater-Zyberk†

Institute of Rheumatology, Prague, \*Institute of Microbiology, Novy Hradek, Czech Republic, †SeroPharmaceutical Research Institute, Geneva, Switzerland.

**Introduction:** Inflammatory cell infiltration and synovial activation are important processes in rheumatoid arthritis. Chemotactic gradients of various chemokines are responsible for cell attraction and possibly for their activation. We have previously detected strong expression of chemokine receptor CXCR3 in the rheumatoid joint by immunostaining.

**Aim:** Characterization of the cells expressing CXCR3 in RA synovial membrane.

**Methods:** Synovial tissue samples were obtained from RA patients undergoing synovectomy or a total joint replacement. Cells were

released by digestion with collagenase, DNase and briefly with hyaluronidase. A three colour fluorescence analysis was performed with FITC conjugated anti-CXCR3 mouse MAb (R&D) and with a panel of phycoerythrin (PE) conjugated MAb (anti-CD3, CD4, CD8, CD19, CD55, CD31, CD68, CD14 and CD45). Live cells were identified by propidium iodide. PBCs were stained using the same protocol.

**Results:** As expected, a proportion of CD3+ and CD4+ blood and synovial cells were CXCR3 positive. In addition, CXCR3 was also seen in synovial cells positive for CD55, CD14, CD8 and to a lesser extent CD31. However, in contrast to the surface staining of cells from peripheral blood, synovial cells displayed only intracellular staining for CXCR3. No CXCR3 staining could be detected on the surface of any type of viable synovial cell, including CD3 positive lymphocytes.

**Conclusions:** Flow cytometry identifies synovial cells that display intracellular CXCR3 staining. These cells are comprised of T lymphocytes, macrophages, possibly synovial fibroblasts and endothelial cell populations. The intracellular presence of CXCR3 suggests a possible internalization of this molecule, which may be a consequence of ligand binding. The significance of this phenomenon and of CXCR3 expression in cell types other than leukocytes remains to be determined.

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## P22

### Interleukin-13 (IL-13) in autoimmune rheumatic diseases: relationship with autoantibody profile

T Rinaldi, A Spadaro, V Riccieri, E Taccari and G Valesini

Dipartimento di Terapia Medica, Unità di Reumatologia, Università "La Sapienza", Rome, Italy

The production of rheumatoid factor (RF) and antinuclear antibody by B-cells could depend on different cytokines action. We evaluated IL-13 serum levels in 230 patients with autoimmune rheumatic diseases including rheumatoid arthritis (RA) [M/F=22/62; mean age=55.2 (25-76) yrs; mean disease duration = 116 (5-605) months], SLE [M/F=17/97; mean age=38.3 (15-70) yrs; mean disease duration = 77 (1-456) months], Sjögren's syndrome (SS) [M/F=2/50; mean age = 55.2 (26-81) yrs; mean disease duration = 82 (3-540) months], and systemic sclerosis (ScS) [M/F = 1/31; mean age=50.6 (20-73) yrs; mean disease duration = 113 (12-276) months], in order to investigate the relationship of this cytokine with the autoantibody profile.

Serum levels of IL-13 (pg/ml) were significantly increased in patients with RA ( $P < 0.00003$ ), with SLE ( $P < 0.03$ ), with SS ( $P < 0.0007$ ), with ScS ( $P < 0.025$ ) as compared to controls. IL-13 serum levels correlated with those of RF in RA ( $P < 0.00001$ ), SLE ( $P < 0.003$ ) and ScS ( $P < 0.03$ ). IL-13 levels were higher in RA ( $P < 0.0002$ ), SLE ( $P < 0.004$ ) and ScS ( $P < 0.05$ ) patients with RF than patients without RF. SS patients with anti-SSA/Ro antibodies had significantly higher IL-13 levels than SS patients without this autoantibody ( $P < 0.036$ ). No statistically significant correlation was found between IL-13 levels and any other antinuclear autoantibody or total immunoglobulin levels or main clinical features of each disease.

This study suggests that IL-13 may be involved in the pathogenesis of autoimmune rheumatic diseases, with a relevant role on RF production. In SS, the lack of correlation between IL-13 and RF is probably due to the peculiar characteristics of this antibody in the disease. We can conclude that the mechanisms involved in RF synthesis recognise different pathways depending on the underlying autoimmune disease.



arthritogenic CW only by 21-34%, whereas the nonarthritogenic CW was degraded by 77-78%, both after 24h incubation. Furthermore, degradation by mutanolysin significantly increased the capacity of the arthritogenic PG to stimulate rat macrophages to secrete TNF- $\alpha$  and MCP-1, whereas it dramatically decreased such a capacity of the nonarthritogenic PG, suggesting that peptides with proinflammatory activity are released from the arthritogenic PG. These results, obtained with an arthritogenic and nonarthritogenic strains of *E. aerofaciens*, indicate that capacity to resist biodegradation, leading to persistence in the tissues, and to release proinflammatory PG peptides, are crucial factors determining arthritogenicity or nonarthritogenicity of a bacterial CW.

## P27

### Immune complex stimulation of peripheral blood mononuclear cells result in enhancement of suppression of IL-12 production dependent on soluble serum factors

A Tejde, K Nilsson Ekdahl, B Nilsson and J Rönnelid

*Department of Clinical Immunology, University Hospital, Uppsala, Sweden*

Immune complexes can induce the production of various cytokines in vitro. Both IL-10 and IL-12 could be induced by addition of heat-aggregated immunoglobulins to mononuclear cells in serum-free cell culture systems. Addition of native serum to the cell cultures influenced the effects on IL-10 and IL-12 in opposite ways. While IL-10 levels were increased in cell cultures with native human serum, IL-12 production was inhibited as compared to cultures with monomeric IgG. Two series of experiments suggested that the effects of immune complexes on IL-12 production depended on the activity of the classical complement pathway in the serum: 1.) Heat-inactivation of serum reverted the inhibitory effect of immune complexes on IL-12 production. 2.) C4 deficient serum behaved as a heat-inactivated normal serum concerning the effects on IL-12 production, and this effect could be reversed by addition of C4. The effects of neutralizing IL-12 had modest effect on immune complex-induced IL-10 production, and the effects of neutralizing IL-10 had no effect on IL-12 production. IL-10 production in the presence of immune complexes could be partially blocked by anti-Fc $\gamma$ RII antibodies, while the immune complex-mediated effects on IL-12 not changed by blocking Fc $\gamma$ RII or Fc $\gamma$ RIII. Opposite and complement-dependent effects on the production of IL-10 and IL-12 can be of importance in cytokine-dependent autoimmune diseases like rheumatoid arthritis or systemic lupus erythematosus, where local or systemic activation of the classical complement pathway participate in the disease processes. Blocking of complement activation or receptors for activated complement components might gain increased attention as potential targets for immune therapies in the light of such cytokine-deviating effects.

## P28

### Inflammatory arthritis, hypoxia and vascularity

PC Taylor, A Steuer, P Etherington, D Cosgrove and RN Maini

*Kennedy Institute of Rheumatology at Imperial College School of Medicine, London, W6 8LH, UK*

We have employed novel technology to investigate the relationship between synovial tissue oxygen levels and vascularity in human inflammatory arthritis. Silver microelectrodes were used to measure

synovial tissue oxygen levels in knee joints of 15 patients with inflammatory arthritis (6 RA, 2 SLE, 1 psoriatic, 1 crystal, 2 reactive and 2 seronegative oligo-arthritis). Synovial membrane cells were obtained from tissue biopsies and *ex-vivo* production of vascular endothelial growth factor (VEGF) was measured. In RA patients, 50 ml N/Saline was injected into the joint and the electrode positioned in the cavity such that the rate of oxygen consumption could be measured. Microelectrodes were also used to assess synovial pO<sub>2</sub> levels in a single metacarpophalangeal (MCP) joint in 5 RA patients. These joints were simultaneously imaged by high resolution ultrasound and power colour doppler to determine the relationship between joint architecture, vasculature and tissue pO<sub>2</sub>.

In knees, synovial tissue pO<sub>2</sub> levels were significantly lower in patients with active RA (mean = 7 mm Hg) than in patients without RA (mean = 40 mm Hg;  $P = 0.002$ ). In RA, oxygen was consumed from N/Saline introduced into the cavity at a rate of 20.5 mm Hg/min. Production of VEGF from synovial cells was greater for patients with RA (mean = 868pg/106 cells) than from synovial cells from patients without RA (mean = 84pg/106 cells;  $P < 0.01$ ).

In the 5 MCP joints studied, a total of 9 vascular areas were sampled. The mean pO<sub>2</sub> at these sites was 97 mmHg. In 19 non-vascular areas sampled, the mean pO<sub>2</sub> was 34 mm Hg (range 6-73). In a vascular erosion the tissue pO<sub>2</sub> was measured as 41 mm Hg.

In conclusion, marked hypoxia is observed in selected regions of inflamed synovium and is a likely stimulus for local VEGF production and angiogenesis. However, the increased vascularity associated with erosive damage is insufficient to restore oxygen homeostasis at the site of joint destruction.

## P29

### Cartilage-derived morphogenetic protein-1 and -2 are endogenously expressed and stimulate proteoglycan synthesis in healthy and osteoarthritic human articular chondrocytes

K Bobacz, A Soleiman\*, W Graninger and L Erlacher

*Department of Rheumatology, University of Vienna, Austria;*

*\*Department of Pathology, University of Vienna, Austria*

**Objective:** Cartilage-derived morphogenetic protein-1 and -2 (CDMP-1 and -2) form a subgroup within the Bone morphogenetic protein family. While they are essential during embryonic joint and limb development, their role in postnatal articular cartilage is not fully clear. In this study we examined the stimulatory effects of CDMP-1 and -2 on proteoglycan synthesis and cell proliferation on postnatal human articular chondrocytes and investigated the hypothesis that osteoarthritic chondrocytes lose their responsiveness to CDMP-1 and -2 compared to healthy cells and thus lead to a decrease in proteoglycan synthesis that impairs maintenance of matrix integrity.

**Methods:** Chondrocytes were isolated from human articular cartilage from patients with and without osteoarthritic lesions. Cell number was assessed directly after collagenase digestion and chondrocytes were cultured as monolayers for a period of seven days in a chemically defined serum-free basal medium (BM) with and without the addition of recombinant CDMP-1 and -2. The proteoglycan-synthesis rate was measured by [<sup>35</sup>S]sulfate incorporation into newly synthesized macromolecules. Growth factors influence on cell proliferation was investigated by [<sup>3</sup>H]thymidin incorporation. Using RT-PCR the endogenous expression of CDMPs and their respective type I and type II receptors was examined.

**Results:** Cell number per mg tissue of osteoarthritic cartilage was significantly reduced, on an average by 45%, compared to healthy controls ( $P < 0.007$ ). CDMP-1 and -2 stimulation markedly



(pooled odds ratio, 2.53 [1.37-4.70]). Stratification further revealed a possible association of carriage of C-1-2-5\*192 with protection from SLE beyond the effects of HLA-DR3 and TNF-308A. Gene dose effect was observed for -308A only (homozygotes, 7.75[3.01-20.0], heterozygotes, 3.15[1.85-5.37]). In multivariate analysis, the association between HLA-DR3, TNF-308A, and C-1-2-5\*192 remained independently associated with susceptibility to SLE (2.58 [1.29-5.18], 2.76 [1.43-5.31], and 0.26[0.10-0.66], respectively).

**Conclusion:** An association of carriage of TNF-308A with susceptibility to SLE can not be attributed to linkage to HLA-DR3, nor to other polymorphic markers in the vicinity of the TNF gene. Further loci that are independently associated with SLE might be located in the vicinity of marker C-1-2-5.

**P33**

**A single nucleotide polymorphism on the IL-10 locus defines an expression polymorphism and a possible risk factor to develop RA**

**LR Lard, JJM Schonkeren, E Pieterman, R Westendorp\*, FC Breedveld and TWJ Huizinga**

*Departments of Rheumatology and \*Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands*

**Introduction:** IL-10 production differs between individuals. We have evaluated the IL-10 production in whole blood cultures with/without LPS. The comparison of monozygotic twins, sibs and unrelated individuals yielded an estimate of heritability of 70% (Lancet 98). Moreover the ex-vivo IL-10 production was associated with haplotypes defined by alleles of CA-repeats (PNAS 98). In line with these results we have demonstrated that the interindividual differences in production of mRNA encoding IL-10 are similar than the interindividual differences in IL-10 protein production (Rheumatology 2000).

**Aim:** A) to define SNPs associated with common haplotypes. B) to study the association of IL-10 production with haplotypes/SNPs. C) to measure the distribution of IL-10 SNPs in RA versus controls. **Methods:** DNA of high and low IL10 producers were sequenced. Subsequently, the association between LPS-induced IL-10 production and previously described (Lancet 98) panel was analyzed. **Results:** The following SNPs were identified: -3575 A to G, -2849 A to G, -2763 A to C and -1330 A to G. Previously (Genes and Immunity 99) we have identified 4 ancestral IL-10 haplotypes. The current SNP's on: IL10.1 R3-AAAA-(IL10G)-GCC, IL10.2 R2-TGCG-(IL10G)-ACC, IL10.3 R2-AGAA-(IL10G)-GCC, and IL10.4 R2-TGCC-(IL10G)-ATA. To investigate whether these SNP's were functional we analyzed the LPS-induced IL-10 production of 161 healthy donors with a specific genotype: -3575: AA (n = 38) 1896 ng/ml, AT (n = 76) 3232 ng/ml, TT (n = 47) 3195 ng/ml. -2849: AA (n = 21) 2115 ng/ml, AG (n = 75) 2950 ng/ml, GG (n = 65) 3111 ng/ml (Mann-Whitney test both P < 0.05). Next, the analysis was repeated in a different group of donors: 135 partners of patients with SLE/MS: -3575: AA (n = 29) 4190 ng/ml, AT (n = 71) 4521 ng/ml, TT (n = 35) 4401 ng/ml (MW-test P = 0.6).

-2849: AA (n = 26) 3845 ng/ml, AG (n = 41) 4577 ng/ml, GG (n = 68) 4543 ng/ml (MW-test P = 0.04, for G carrier versus non G: P = 0.02). Next, the distribution of -2849 SNP was compared in RA patients compared to controls. Control-Panels were 1) partners of MS-SLE patients (n = 135) and 2) organ donors (n = 168). RA-patients were: 1) incident RA cases, 2) outpatient consecutive RA and 3) RA patients from our early arthritis cohort.

**Conclusion:** We (Eskdale et al, Lancet 98) have previously found that the allele IL-10R3 microsatellite was less frequent in three ethnic groups of RA patients (Afro-americans P < 0.01, English P < 0.008 and Scottish P < 0.02). The SNP that defines the haplotype on which R3 is located is also less prevalent in three groups of

	Control		RA Patients		
	(1)	(2)	(1)	(2)	(3)
AA	27	16	3	24	16
AG	42	64	38	141	74
GG	71	88	51	152	91

Total chi-square: P = 0.0014

dutch RA compared to two groups of dutch controls. These data suggest that a high innate IL-10 production is a risk factor for RA. This may be due to the B-cell stimulating properties of IL-10.

**P34**

**Elevated levels of soluble intercellular adhesion molecule -1 in systemic lupus erythematosus**

**N Kijukvina, S Shekshina, E Alexandrova, A Novicov and E Nassonov**

*Moscow Medical Academy, Institute of Rheumatology of RAMS, Russia*

**Objective:** To assess the value of measuring serum levels of soluble intercellular adhesion molecule 1 (sICAM-1) in systemic lupus erythematosus (SLE).

**Material and methods:** We studied 35 patients (pts) (7 female, 28 male), satisfying the ACR criteria for SLE. Mean age of pts was 31,4±12,0 years (range 17-63), mean disease duration was 81,8±70,5 month (range 2-240). Disease activity was assessed by disease activity indices (SLAM, SLEDAI). Enzyme-linked immunosorbent assay was used to measure levels of sICAM-1 (R&D, USA). The results were compared with 18 healthy subjects.

**Results:** Levels of sICAM-1 were found elevated (more than 2 SD above the mean in normal controls, 443 ng/ml) in 7 of 35 (20%) pts with SLE. The relations between positive sICAM-1 and some clinical manifestations of SLE have been detected. We found significant correlation between individual sICAM-1 serum level and the SLEDAI (r=0.43) and SLAM (r=0.56) scores, and ESR (r=0.53).

Parameters, (%) or mean ±SD	Positive sICAM-1 (n = 7)	Negative sICAM-1 (n = 28)	P
ICAM-1, ng/ml	512,3±45,5	284,3±85,0	<0,001
Malar rash	28,6%	14,2%	NS
arthritis	42,8%	25%	NS
nephritis	57,2%	32,1%	NS
CNS involvement	71,4%	39,3%	NS
Serositis	42,8%	14,3%	NS
ESR, mm/h	38,0±24,8	21,2±16,1	<0,05
SLAM, score	16,0±7,4	9,03±5,7	<0,05
SLEDAI, score	18,0±12,4	10,2±7,75	<0,05

**Conclusion:** Elevated serum levels of sICAM-1 can be found in SLE and correlate with disease activity. Longitudinal studies may establish their clinical value in the monitoring or the prognosis of patients.



### P38

#### Redox-sensitive changes in conformation and cellular localization of LAT and downstream TCR signaling lead to hyporesponsiveness of synovial fluid T cells in rheumatoid arthritis

SI Gringhuis, PHJ Remans, EAM Papendrecht-van der Voort, A Leow, EWN Levarht, FC Breedveld and CL Verweij

Department of Rheumatology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands

In rheumatoid arthritis (RA), the synovial fluid (SF) T lymphocytes present in the inflamed joints, display hyporesponsiveness upon engagement of the TCR/CD3 complex despite phenotypic evidence of former activation. We have previously shown that the central and crucial adaptor protein LAT (linker for activation of T cells), which plays a central and crucial role in the T cell receptor (TCR)-mediated signaling pathways, exhibits deficient phosphorylation due to displacement of the integral membrane protein from the plasma membrane in SF T lymphocytes. SF T lymphocytes exhibit several features of chronic oxidative stress, e.g. severely decreased intracellular levels of glutathione (GSH), and our previous studies have indicated that the subcellular localization of LAT is sensitive to changes in the intracellular GSH levels. The cysteine-to-serine substitutions of several cysteine residues (C26/29 or C117) within LAT creates LAT mutants that are resistant to reduced intracellular GSH levels and remain membrane-anchored in GSH-depleted cells.

In this study, we have used the redox-insensitive LAT mutants to study the effect of redox balance alterations, like in SF T lymphocytes, on TCR signaling pathways downstream from LAT and on CD28 signaling pathways. In co-transfection experiments, we show that the presence of the redox-insensitive LAT mutants allows for the partial restoration of the TCR-mediated signaling pathways, but not the signaling pathways induced through the CD28 receptor. The data are indicative that the Raf1-ERK and the calcium-calmodulin pathways leading to transcriptional activation of AP-1 and NFAT, respectively, are very sensitive to reduced intracellular GSH levels, while the activation of the p38/Mpk2 pathway leading to AP-1-mediated transcription is mostly unaffected by chronic oxidative stress. A very proximal event in the CD28-mediated signaling pathways seems to be extremely sensitive to GSH depletion since costimulation did not affect the transcriptional activity of either AP-1 or NF- $\kappa$ B.

We conclude that the signaling pathways in SF T lymphocytes from RA patients are affected at several levels by chronic oxidative stress, all contributing to the observed hyporesponsiveness of these cells.

### P39

#### Peripheral corticotropin releasing hormone signaling is mediated by Type 1 $\alpha$ receptors in early human inflammatory arthritis

A McEvoy, B Bresnihan, O FitzGerald and E Murphy

Department of Rheumatology, St Vincent's University Hospital, Dublin, Ireland

Corticotropin Releasing Hormone (CRH) is essential for modulating the effects of the inflammatory response *in vivo*. Elevated levels of CRH are produced locally in inflamed human synovial tissue and observations indicate a role for CRH in the pathogenesis of inflammatory joint disease. CRH action is initiated by two distinct subtypes of CRH receptors, CRH-R1 and CRH-R2, which are approximately 68% homologous. Each subtype exhibit spliced vari-

ants ( $\alpha$  and  $\beta$ ), displaying pharmacologically and functionally distinct isoforms.

To further elucidate the peripheral biological role for CRH we examined the expression of known CRH receptor subtypes in inflamed human synovium ( $n = 14$ ) and compared the expression patterns to normal synovium. Immunohistochemistry and RT-PCR confirmed enhanced expression of CRH-R1 receptors in rheumatoid (RA) and psoriatic (PsA) arthritis synovial tissue. In all tissues studied CRH R1 $\alpha$  mRNA was identified, however, we were unable to detect other CRH R1 or CRH R2 isoforms in the same cohort of patients. Immunoreactive CRH-R1 is abundantly expressed on vascular endothelial cells and discrete perivascular cell populations, positively identified as mast cells. In contrast, in normal synovial tissue, neither CRH receptor subtype is expressed.

Selective up-regulation of CRH receptors in inflamed synovial tissue indicates that CRH functions locally, in an autocrine/paracrine receptor-mediated response. Our findings suggest that CRH signaling, via CRH-R1 $\alpha$ , may play a role in both vascular changes and pathologic mechanisms associated with joint inflammation.

### P40

#### Detection of the "Kreiser" (maf B) gene by combination of in situ-hybridization and immunohistochemistry of RA-, osteoarthritis- and normal controls-synovial tissue samplings as a potential significant marker for early RA

U Vigna\*, B Ostendorf\*, T Pauly\*, T Giel\*, U Jeffrey†, R Murray†, M Schneider\*

\*Multipurpose Arthritis Center, Heinrich-Heine University Duesseldorf, Germany; †EOS-Biotechnology, San Francisco, USA

**Introduction:** By analyzing gene expression profiles of arthritic tissue on DNA microarrays (EOS) compared to the "Body Atlas", a reference database of 13 normal human tissues, we found the RAB3 "Kreiser" (maf B) gene (member of the maf gene family and encoding for a transcription activator specific for mesenchymal and neuronal organogenesis) highly expressed in early rheumatoid arthritis (RA) (< 2 years disease duration).

**Objective:** To investigate the functional "Kreiser" gene expression in RA-, osteoarthritis- and normal controls- synovial samplings by combination of in situ-hybridization and immunohistochemistry.

**Methods:** We analyzed synovial biopsies ( $n = 12$ ; 7f/5m) from 5 RA- (3 early RA, 2 RA), 4 osteoarthritis-patients and 3 normal controls, which were taken by arthrotomy by various indications and miniarthroscopy of MCPs (2 early RA). Samples were analyzed by in situ-hybridization with the "Kreiser" (maf B) gene-mRNA and immunohistochemistry (e.g. Ki 67, CD 68).

**Results:** We detected increased "Kreiser"-mRNA levels in 3 early RA samples in the synovial lining layer and no signals in the control and compared samples. At higher concentrations (>1ng/ $\mu$ l) of RNA-oligonucleotides unspecific hybridization-signals prevailed in tissues of all diseases (even in normal controls). The combination of both methods (in situ-hybridization and immunohistochemistry) identifies the single cells inside the synovial lining layer which contains the highly expressed RAB3 "Kreiser" (maf B) gene.

**Conclusion:** Based on the gene expression profiles through oligonucleotid-microchip-array-analysis by EOS and the detection of the increased "Kreiser" (maf B) gene expression in combination of in situ-hybridization and immunohistochemistry of RA-synovial tissue samplings we discuss the "Kreiser"-gene as a potential inducing element in the pathogenesis of early RA. Further serial studies are needed to clarify the significance of "Kreiser" especially for early RA and the molecular pathogenesis of this disease.



**P44**

**Specific suppression of the transcription factor AP-1 by mepacrine**

**KM Stuhlmeier, C Linnert and H Bröll**

*Ludwig Boltzmann Institute for Rheumatology and Balneology, Vienna-Oberlaa, Austria*

Mepacrine has been used for decades and the beneficial effects of this drug are well described. Since endothelial cells (EC) are in many cases the first cells to come in contact with drugs, the effect of mepacrine on certain aspects of EC biology were studied. First, our data demonstrate that at high doses mepacrine can have a marked impact on the integrity of the EC monolayer without grossly interfering with cell viability. The described impact of mepacrine on EC might explain, at least in part, the negative effects of this drug observed in the past. More importantly, mepacrine profoundly effects gene regulation in EC and fibroblasts. Mepacrine binds to DNA in a sequence specific manner. While NF- $\kappa$ B-DNA interactions are not effected, AP-1-DNA binding is blocked by mepacrine. Such differential effects are presumably due to sequence specific intercalation of mepacrine into the AP-1 consensus element. Pre-incubation of oligonucleotides resembling this sequence blocked the subsequent binding of nuclear extract containing AP-1 protein(s). Consequently, mepacrine prevents the upregulation of genes which depend mainly on the activation of AP-1. One of the few genes which have been found to depend heavily on the activation of this transcription factor is metalloproteinase-1 (MMP-1). We demonstrated by western blot that treatment of fibroblasts with mepacrine completely prevented subsequent upregulation of MMP-1. Since MMP-1 plays an important role in the propagation of rheumatic diseases, we suggest that the beneficial effect of mepacrine seen in the past is due, at least in part, to the described mechanisms.

**P45**

**Patterns of differentially expressed genes in synovial tissue from RA and OA patients and from normal joints**

**U Ungethüm, T Häupl, J Zacher\*, A Gursche\*, Förstert, P Reutermann†, A Pruß, V Krenn and G-R Burmester**

*Department of Rheumatology, Charité, Berlin; \*Dept. of Orthopedics, Klinikum Buch, Berlin; †Department of Orthopedics Waldkrankenhaus Bad Dübren; ‡Department of Orthopedics, KMG Kliniken, Kyritz; Germany*

**Objective:** To identify key genes in the pathomechanism of rheumatoid arthritis (RA), synovial tissues from RA, osteoarthritis (OA) and from normal joints (ND) were compared by a subtractive hybridization technique, the representational difference analysis (RDA).

**Methods:** Synovial tissues from 3 RA, 3 OA patients and 5 normal joints were selected according to their disease-characteristic immunohistochemical findings and to their expression of high versus low levels of inflammatory (IL-1 $\beta$ , TNF- $\alpha$ ) and destructive markers (MMP-1, MMP-3) as determined by semiquantitative RT-PCR. Pooled mRNA from RA, OA and normal tissues was transcribed, digested by a 4-base-cutter, ligated to adapter-primers and amplified to form representational amplicons. Subtractive hybridizations were performed by different protocols: 1. the OA amplicon (driver) was subtracted from the RA representation (tester); 2. the RA (driver) from the ND (tester) and 3. the ND (driver) from the OA representation (tester). Using primers specific for the corresponding tester, the difference-products were selectively amplified, cloned, sequenced and compared to published sequences in the Genebank. Differential expression of identified genes was validated by semiquantitative RT-PCR.

**Results:** Approximately 150 genes were found to be differentially expressed in RA synovial tissue as compared to OA or ND tissues respectively, or in OA tissues as compared to ND. Interestingly, some genes were identified to be overexpressed in both groups: RA (i.e. difference-product from RA minus OA) and OA (OA minus ND), indicating rather an association to general joint destruction than to RA-specific mechanism. Other genes were found to be differentially expressed only in the RA representation. 30 of the differentially expressed genes identified from each disease group were analyzed in synovial tissues from further 20 RA, 20 OA patient and 20 normal joints. The expression of some genes showed either a significant correlation to those of inflammatory genes (IL-1 $\beta$  and TNF- $\alpha$ ) or to those of destructive markers (MMP-3).

**Conclusions:** The analysis of differential gene expression in chronic joint diseases is a promising approach to identify deregulation of the inflammatory network to explain the inappropriate immune response with autoaggressive outcome. Furthermore a pattern of genes is generated which is specifically or preferentially expressed in RA. Such patterns will be of diagnostic value, especially for disease characterization, longitudinal studies and analysis of therapeutic effects.

**P46**

**MMP-1, MMP-3 and MMP-10 are involved in the degradation of cartilage**

**TCA Tolboom\*, E Pieterman\*, WH van der Laan†‡, AL Huidekoper\*, RGHH Nelissen‡, FC Breedveld\* and TWJ Huizinga\***

*\*Departments of Rheumatology and †Orthopaedic Surgery, Leiden University Medical Centre, Leiden; ‡Gaubius laboratory, TNO Prevention and Health, Leiden, The Netherlands*

Rheumatoid arthritis (RA) is characterised by degradation of cartilage and invasion of fibroblast-like synoviocytes (FLS) into adjacent cartilage. Several families of proteinases are involved in the degradation of cartilage, especially the matrix metalloproteinases (MMP's) and cathepsin K. However, it is not known which MMP's are responsible for the degradation of cartilage and the invasiveness of FLS. In this study, the expression of MMP's 1 to 20 and cathepsin K in cultured FLS obtained from joint replacement surgery from RA, osteo-arthritis (OA) and other non-destructive arthropathies are investigated and compared to the invasiveness of the FLS in a matrigel transwell system. In this matrigel transwell system previous studies have shown that FLS from RA patients were significantly more invasive than FLS from patients with OA or other non-destructive arthropathies.

FLS from synovial tissue of 32 RA, 18 OA and 14 patients with other non-destructive arthropathies were obtained from joint replacement surgery. The FLS were grown to confluency and RNA was isolated at passage 1 or 2. cDNA was synthesized using oligo-dT and reverse transcriptase. Expression of MMP's and cathepsin K was investigated using RT-PCR. For MMP's 2, 3, 7-12, 14-17, 19, 20 and cathepsin K RT-PCR was performed with primers for the MMP under investigation and primers for beta-actin in one mix. For MMP-1 and 13 no primers for beta-actin were in the mix. The intensity of the bands were compared and given a number from 0 (no expression) to 3 (intensity more than beta-actin). These numbers were related to the invasiveness (number of cells) in a matrigel transwell system.

FLS that expressed MMP-1, MMP-3 or MMP-10 were significantly more invasive (median number of invasive cells: 3970, 4525, 4998, respectively) than cells that did not express MMP-1, MMP-3 or MMP-10 (1826,  $P = 0.02$ ; 3081,  $P = 0.01$ ; 2537,  $P = 0.01$ , respectively). Expression of the other MMP's and cathepsin K did not show a significant relationship with invasive growth. Expression



**Results:** The analysis of CD19+ B cell frequencies of RA patients revealed a bimodal distribution in the study population separating one group of patients with B cell counts below 8.5 % of all lymphocytes (B cell low patients, 62 % of the study population) from a second group with more than 8.5 % B cells (B cell high, 38 %). HLA genotyping revealed, that the two groups were immunogenetically distinct. B cell low patients were more frequently SE positive than B cell high patients (84.5 % vs. 50 %,  $P < 0.001$ ), and SE positive patients had lower CD19 percentages in the rank-sum analysis when compared to SE negative ones (6.3 % vs. 14.0 %,  $P < 0.001$ ). Comparative analysis of a healthy control group showed, that B cell frequencies were diminished in SE positive and increased in SE negative patients.

B cell low patients were found to have significantly lower concentrations of RF IgM, RF IgA, and serum IgM, but not of serum IgG, when compared to the B cell high group. Multivariate analysis revealed the presence of low B cell counts to be associated with the presence of the shared epitope sequence, RF IgM seronegativity and low concentrations of serum IgM, but not with disease activity, gender, age at disease onset or disease duration.

**Conclusion:** We have found a diminished size of the peripheral B cell pool in SE positive RA patients, that is associated with lower RF IgM titers and a suppression of the parameters of polyclonal IgM, but not IgG secretion. Suppression of polyclonal autoreactivity in SE positive RA patients by clonal deletion of autoreactive, IgM+ B lymphocytes is one possible explanation for decreased B cell counts in RA.

## P51

### In adjuvant-induced arthritis the disease-triggering adjuvant squalene accumulates in draining lymph nodes but not affected joints

BC Holm\*, L Svelander\*, A Bucht\*\* and JC Lorentzen\*\*

\*Department of Medicine, Unit of Rheumatology, Karolinska Institute, Stockholm, Sweden; †Department of Biomedicine, Division of NBC Defense, Defense Research Establishment, Umeå, Sweden;

‡Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden

Nonspecific stimulation of the immune system by adjuvants can cause joint-specific inflammation in rats, as exemplified by arthritis induced with the endogenous cholesterol precursor squalene (C30H50). To determine the uptake and distribution of injected adjuvant, and more specifically to determine whether adjuvant accumulates in affected peripheral joints, tritium-labelled squalene was used to induce arthritis in arthritis-prone DA rats. All organs, including hind paws and the site of injection, were collected at different stages of disease development. The deposition of oil was subsequently quantified by dissolving the tissues followed by scintillation counting.

The majority of injected oil never leaves the injection site, and no adjuvant oil is accumulated in the peripheral joints. Organ samples taken early prior to clinical disease and after arthritis onset displayed a similar distribution of oil, except for the draining lymph nodes and the intestines. In the draining lymph nodes, the deposition of oil accumulated over time, whereas the reverse was the case for the intestines.

A passive transfer of squalene-induced arthritis with lymph node cells was successfully accomplished, both with cells from draining inguinal lymph nodes and cells from lymph nodes not draining the injection site (axillary). Since uptake of squalene was minimal in axillary lymph nodes, this result indicates that the oil need not be present for passive transfer of the disease.

In conclusion, we report an accumulation of the arthritis triggering squalene in the draining lymph nodes but not in the peripheral joints

from the time of injection to the disease onset. This uptake evokes a systemic immune activation of unknown mechanisms that subsequently lead to a joint specific inflammation.

## P52

### p205 induces the production of rheumatoid factors

F Schumann\*, U Ungethüm\*, S Adelt\*, H Hofseß\*, A Gursche†, J Zacher†, JB Natvig‡, J-M Engel§, G-R Burmester\* and S Bläß\*

\*Department of Rheumatology & Clinical Immunology, Charité University Hospital, Berlin; †Orthopedic Clinic Berlin Buch; ‡Institute of Immunology, Rijkshospitalet Oslo, Norway; §Rheumaklinik Bad Liebenwerda, Germany

The p205 autoantigen is the strongest stimulatory antigen for T cells known in rheumatoid arthritis (RA). It contains an 11 aminoacid stretch identical to a sequence (278-288) located in the CH2 domain of immunoglobulin G. This domain contains the major epitopes of rheumatoid factors. This study aimed to analyze if the p205-specific T cell responses are also directed against RF epitopes and to analyze the role of p205 in the production of rheumatoid factors in general.

p205 was enriched from synovial fluid as described earlier. p205-derived peptides were chemically synthesized. T cell proliferation assays were performed with cells obtained from RA and control patients and healthy individuals.

Sequencing and mass spectrometry by matrix assisted laser desorption-time of flight (MALDI-TOF) of p205 revealed that it contains sequences with similarity and identity to IgG and other members of the immunoglobulin superfamily. p205 was detected in the synovial membrane of RA patients by antisera specific for p205-derived peptides. Cells staining positive for p205 were also positive for the macrophage marker CD68. p205 staining did never occur in B cell clusters staining positive for CD19 or in T cell infiltrates staining positive for CD3. No B and T cells were detected in the highly p205-positive lining and sublining of the synovial membrane. p205 could react with monoclonal rheumatoid factors (RF). Those RF that reacted also with p205 tended to be of a binding specificity characteristic of RA. Those RF that did not react with p205 tended to be of a binding specificity that is also observed in healthy immunized donors or patients with Waldenström's macroglobulinemia.

Synovial fluid (SF), SF-derived p205 and p205-derived peptides were used as antigens in T cell proliferation assays. As control antigens, a mock peptide and PHA were used. SF, p205 and p205-derived peptides stimulated T cells from two thirds of RA patients, but not from patients with other rheumatic diseases or from healthy individuals. SF, p205 and the 11aa p205 peptide with sequence identity to IgG were extremely high stimulators of proliferation in the majority of RA patients and were often in the range of the mitogen PHA. Two other p205-derived peptides were also stimulatory for RA-derived T cells, but to a lesser degree and at a lower frequency of patients. None of these peptides induced T cell proliferation in patients with other rheumatic diseases or healthy individuals. No reactivity was observed with the mock peptide in any of the patients. T cells specific for p205 cocultured in the presence of IgG-specific B cells induced the production of rheumatoid factors upon stimulation with cognate antigen and the 11mer peptide 3. RFs could also be induced upon immunization of rabbits with peptide 3.

p205 is a major target of autoreactive T cells in RA and appears to be a novel member of the immunoglobulin superfamily. It contains an IgG-identical stretch and p205 is targeted by RFs. The IgG-identical peptide 3 stimulates T cells such that they can provide cross-help for RF-secreting B cells *in vitro* and *in vivo*. p205 may thus likely be the trigger of RF production in RA and may thus be of pathogenic importance.



**P56**

**Immune response to hn and snRNP in autoimmune mice. A model for the development of lupus autoimmunity by a single initiator T helper epitope?**

**F Monneaux, H Dumortier, J-P Briand, G Steiner\* and S Muller**

*UPR 9021, CNRS, IBMC, Strasbourg, France; \*Vienna University, Vienna, Austria*

Systemic lupus erythematosus is characterised by the presence of high titers of autoantibodies reacting with various components of the small and heterogeneous nuclear ribonucleoprotein particle. It has been suggested that these antibodies are produced by an antigen-driven mechanism under the dependence of antigen-specific T cells. To investigate the role of T cell help in this process, we sought with twenty overlapping peptides the Th epitopes on the U1-70K snRNP in unprimed H-2<sup>k</sup> MRL/lpr lupus mice and immunised CBA normal mice. The peptide 131-151 was recognized by both IgG autoantibodies and CD4<sup>+</sup> T cells from 7-9 week-old MRL/lpr mice. In this test, APCs from MRL/lpr mice were required, APCs from naive CBA mice failed to stimulate CD4<sup>+</sup> cells from MRL/lpr mice. Peptide 131-151 bound both I-A<sup>k</sup> and I-E<sup>k</sup> class II molecules and favoured an IL-2 positive T cell response but not IFN- $\gamma$ , IL-6 and IL-10 secretion. Segment 131-151 is localised within the RNP80 motif and contains residues that are highly conserved in many nuclear, nucleolar and cytoplasmic RNA binding proteins. In parallel, we studied the Ab response to the A2/B1 hnRNP in different murine models of lupus, and found in residues 50-70 a major epitope recognized very early during the course of the disease by Abs from most of MRL/lpr mice. Peptide 50-70 generated in CBA/J mice an effective Th cell response with IL-2 and IFN- $\gamma$  secretion. Interestingly, this peptide also contains the highly conserved sequence present in peptide 131-151 of the 70K protein. It is possible that starting from a single Th epitope, the sequence of which is repeated in several self-proteins involved in the same complex or close cellular components, a larger, diversified Th response is generated, which extends via intra-and inter-molecular spreading of the T and B cell responses.

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**P57**

**Persistence of plasma cells in the kidneys of autoimmune NZB/W mice**

**G Cassese\*, S Lindenau\*, B de Boer\*, S Arce\*, A Hauser\*, G Riemekasten†, C Bereke\*, F Hiepe†, A Radbruch\* and RA Manz\***

*\*Deutsches Rheuma-Forschungszentrum, Berlin, Germany;*

*†Department of Medicine, Rheumatology and Clinical Immunology, Charité University Hospital, Humboldt University, Berlin, Germany*

NZB/W mice develop a disease similar to human systemic lupus erythematosus (SLE), including autoantibody production, hypergammaglobulinaemia and inflammation of the kidneys. It is known that large numbers of lymphocytes infiltrate the kidneys of these mice but the role of this organ for the production of antibodies is not clear. Here, we compare the role of bone marrow, spleen and

inflamed kidneys of NZB/W mice for the activation of B cells and for the persistence of antibody secreting cells (ASC). ASC are present in the kidneys of mice with full blown disease, as many as in the spleen and bone marrow, and 50 times more than in the kidneys of normal mice. In the kidneys, ASC are located mainly in the outer medulla, close to B- and T cell infiltrates. The specificity of the ASC in the inflamed kidneys is not restricted to self-antigens. After immunization of NZB/W mice with Ovalbumin (OVA), the antigen-specific ASC are found initially exclusively in the spleen. Weeks later, during a period of at least 3 months, OVA-specific ASC are found in stable and high numbers within the bone marrow and the kidneys of these mice, but no longer in the spleen. As determined by FACS, B cells with a germinal center phenotype (B220<sup>+</sup>/PNA<sup>+</sup>) are found only in very low numbers in the kidneys, but in high numbers in the spleen of NZB/W mice. By histology, germinal centers could not be detected in the kidneys, but in the spleen. The lack of B cell activation and the kinetics of the appearance of OVA-specific ASC suggest that in autoimmune NZB/W mice kidneys, plasma cells generated during an immune reaction in secondary lymphoid organs, later accumulate and persist, like in bone marrow. These experiments identify the inflamed kidneys of NZB/W mice as site of prime relevance for the homeostasis of plasma cells, irrespective of their specificity, suggesting that chronically inflamed tissue attracts plasma cells as such and extends the overall capacity of the body for plasma cells, allowing autoreactive plasma cells to survive for long times within the inflamed tissue and to provide exorbitant titers of antibodies locally.

**P58**

**In vivo preactivated autoreactive Th cells in healthy individuals**

**A Radbruch, S Nitsch, B Holzknicht, E Gromnica-Ihle, S Schneider, F Hiepe, A Thiel**

*Deutsches Rheuma-Forschungszentrum Cell Biology, Berlin, Germany*

The direct analysis of autoantigen-specific Th-cells has been hampered so far by the lack of appropriate methods to directly determine their frequency or functional capabilities. We have applied a set of new techniques to directly identify and analyze autoantigen-specific T-cells in both affected and healthy people according to their effector functions (e.g. effector cytokine production) after provocation with antigen.

We have used these technologies to analyze Th-cells specific for SLE-associated autoantigens, in particular nucleosomes and the ribonucleoprotein La. Surprisingly, *in vivo* pre-activated autoantigen-specific Th-cells secreting IFN $\gamma$  and TNF $\alpha$ , could be detected not only in SLE-patients, but also in normal healthy persons, with frequencies ranging from 0.02% to 0.1%. Preactivation of these cells *in vivo* was confirmed by the fact that they expressed CD45RO but not CD45RA. Some of them had down-regulated expression of CD45RB and CD27. We also detected in healthy donors *in vivo* preactivated Th-cell specific for the self-antigen alphaB-Cristallin, a small heat shock protein. Up to 0.5% of peripheral Th cells specifically react with IFN $\gamma$  secretion upon short term stimulation, a hallmark of a recall response, i.e. *in vivo* preactivation.

The fact that *in vivo* pre-activated, autoantigen-specific Th-cells can be detected at comparable frequencies and with similar cytokine secretion patterns in blood of normal persons and patients suffering from a disease in which such Th cells are suspected to play a pivotal role, points to mechanisms other than central and peripheral tolerance that control the initiation of those autoimmune reactions.



**Results:** PBMC pre-incubated for 96 h with medium alone showed a good proliferation to subsequent stimulation with anti-CD3 mab, whereas IL-2 induced only little proliferation. Unresponsive TC fail to produce IL-2 as demonstrated at transcription level by rt-PCR. In contrast, PS cells responded only minimally to subsequent stimulation with anti-CD3, but the addition of IL-2 induced a strong proliferation, comparable to IL-15. Both, PS and NS TC responded well to re-stimulation by PHA, whereas Con A induced proliferation mainly of NS cells and thus had similar effects as anti-CD3. In the presence of 10% freshly isolated MO PS cells were able to respond significantly to subsequent TCR challenge. But the addition of MO from NS cultures to PS-TC did not fully restore proliferation. Interestingly, when cells were allowed to rest for 168 h, the responsiveness of PS lymphocytes was restored. Surprisingly, immunoblots revealed that PS cells had a higher intracellular content of  $\zeta$ -chain and p56lck. Both, PS TC and MO show higher expression of different activation associated surface molecules (HLA-DR, CD25, CD69, and costimulatory molecules).

**Discussion:** Our results show a mechanism leading to a temporary unresponsiveness to TCR ligation of preactivated TC although adequate costimulatory support seems available. The rate limiting events for IL-2 production can be overcome by bypassing the TCR via mitogens or addition of freshly isolated MO. Although we have not been able to fully define the rate limiting events we have been able to exclude various possibilities. TC pre-activated via the TCR can continue to produce and express a variety of molecules such as IFN- $\gamma$ , IL-2R, and cell surface molecules. Thus, their effector function in G1-phase, but not their progression into a mitotic cell cycle seems to be sustained.

## P62

### Effect of CD154-CD40 interactions on collagen production by fibroblasts

VV Yurovsky and B White

University of Maryland and the VA Maryland Health Care System, Baltimore, MD 21201, USA

Interactions of T cells and fibroblasts appear to be important in the development of fibrosis, for example, the restrictive lung disease that follows the inflammatory process of alveolitis in scleroderma (systemic sclerosis, SSc). The intermolecular interactions mediating fibroblast activation are not well characterized. CD154 (CD40 ligand) is an activation-induced T-cell surface molecule which counter-receptor is CD40 expressed on target cells, including fibroblasts. We have found CD154 expression on a number of activated CD8<sup>+</sup> T cell clones derived from bronchoalveolar lavage (BAL) fluids from SSc patients. To begin investigating the potential role of CD154-CD40 interactions in fibroblast activation, we co-cultured CD154<sup>+</sup> Jurkat D1.1 cells or CD154<sup>-</sup> Jurkat E6-1 cells with fibroblast lines derived from dermal biopsies or BAL fluids from SSc patients and control donors. Collagen  $\alpha 2(I)$  mRNA expression in fibroblasts was measured by RT-PCR, with ribosomal protein S9 as an internal standard. Total soluble collagen was measured in co-culture supernatants, using Sircol Bicolor assay. In fibroblasts co-cultured for 6 h with CD154<sup>+</sup> cells, but not CD154<sup>-</sup> cells, normalized collagen mRNA expression and total soluble collagen production were 2 times higher than in fibroblasts cultured alone. Intracellular fluorescent staining did not detect IL-4, IL-10, IFN $\gamma$ , or CD95 ligand expression in either D1.1 or E6-1 cells. These data suggest that CD154-CD40 interactions may enhance collagen production in fibroblasts. As this process continues uncontrolled, it may lead to the development of fibrosis.

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## P63

### Enhanced transendothelial *in vitro* migration of scleroderma lymphocytes

GH Stummvoll, M Aringer, J Grisar, CW Steiner, JS Smolen, R Knobler\* and WB Graninger

Department of Rheumatology and \*Department of Special Dermatology, University of Vienna, Vienna, Austria

**Objective:** T lymphocytes are thought to play an important role in the pathogenesis of Systemic Sclerosis (SSc). Perivascular accumulations of predominantly CD4<sup>+</sup> T-lymphocytes are found at an early stage of scleroderma skin lesions. Moreover, soluble and membrane-bound adhesion molecules are elevated in SSc and may facilitate lymphocyte/endothelial cell contact. To assess the migration qualities of peripheral lymphocytes, we investigated the *in vitro* migration of SSc-lymphocytes through human endothelial cell monolayers.

**Patients and Methods:** Endothelial monolayers were formed by human umbilical vein endothelial cells (HUVEC) in their 3rd to 4th passage seeded on collagen gels and incubated overnight. PBMC were prepared from 6 patients (5f, 1m, mean age 55 $\pm$ 6.5 yr.) fulfilling the ACR criteria for SSc and 6 healthy controls (HC; 5f, 1m, mean age 55 $\pm$ 7.1 yr.). Lymphocyte-migration was measured after one hour of incubation by fractionated harvest of non-adherent, bound, and migrated lymphocytes. Changes in the CD4/CD8 ratio and in the lymphocytic expression of activation markers (CD25, HLA-DR, CD69) and adhesion molecules (CD11a, CD49d) *ex vivo*, during and after migration were investigated by fluorocytometry.

**Results:** The percentage of migrated SSc lymphocytes was increased in each single experiment (Fisher's exact test  $P < 0.03$ ) when compared to HC (9.0 $\pm$ 4.4% vs 5.3 $\pm$ 2.9%). Compared to HC, the CD4/CD8 ratio was only slightly higher in SSc when detected *ex vivo* (2.71 $\pm$ 0.76 vs. 2.22 $\pm$ 0.54,  $P = n.s.$ ), but increased after migration (3.00 $\pm$ 0.57 vs. 1.01 $\pm$ 0.38,  $P < 0.02$ ), whereas the CD4/CD8 ratio in HC fell. The expression of lymphocytic activation markers and adhesion molecules was similar in SSc and HC *ex vivo*. Migrated SSc lymphocytes tended to express higher amounts of CD 25 and CD 49d, but this did not reach statistical significance in our small sample of patients.

**Discussion:** Lymphocyte migration through a human endothelial monolayer is increased in SSc and is accompanied by an increase of the CD4/CD8 ratio. These data suggest that CD4<sup>+</sup> SSc cells are more prone to migration than CD8<sup>+</sup> cells and are in line with the paravascular accumulation of CD4<sup>+</sup> lymphocytes.

## P64

### Autologous dendritic cells stimulate human HSP60 responsive T cells, in the absence of additional exogenous antigen

MS Lillicrap, MK Matyszak, JC Goodall, JL Young, JSH Gaston

University of Cambridge, Dept. of Medicine, Addenbrookes Hospital, UK

**Background:** Animal models and clinical studies of inflammatory arthritis have shown a potentially protective role for autoreactive T lymphocytes recognising the 60 kilodalton heat shock protein (hsp60). The mechanisms of this protection have not been fully characterised. We have previously demonstrated that PBMC from healthy individuals show proliferative responses to human hsp60, and clones have been isolated that recognise the self protein. The objective of the present study was to confirm the autoreactive nature of these cells and determine whether the endogenous antigen could be effectively presented by professional antigen presenting cells.

**Methods and results:** Highly purified recombinant human hsp60 was prepared along with a non-recombinant preparation of mito-



tides. Extremely strict precautions were followed in the clinics and laboratory to prevent contamination. Bacterial DNA could not be detected by PCR with pan 23S rRNA and 16S rRNA in any of the samples. The positive controls, including bacterial DNA and human DNA, were run with each sample, and were always positive. Further, using the same method, 5/15 (33%) synovial fluid samples from patients with *Chlamydia* reactive arthritis were PCR positive. The PCR sensitivity was 2-20 CFU/reaction determined by mixing the living bacteria with ST and using exactly the same experimental procedure as with the patient samples.

Gas chromatography-mass spectrometry has been applied to detect muramic acid (bacterial cell wall specific chemical component) in ST. Preliminary results suggest that low concentration of muramic acid can be detected in the ST from some patients with inflammatory arthritis.

Our results show that bacterial DNA in ST from RA and OA could not be detected by PCR for 23S rRNA and 16S rRNA. Instead, muramic acid could be detected by gas chromatography-mass spectrometry. These observations indicate that the presence of bacterial DNA in ST might not be as prevalent as previously suggested. Nevertheless, the bacterial components may exist in ST.

## Poster Discussion E

### Autoantibodies in CTDs

#### P69

#### Detection of anti-B/B' UsnRNP antibodies in connective tissue disease sera by Western immunoblot

A Ghirardello, A Doria, S Zampieri, D Villalta\*, F Vescovi, PF Gambari

*Division of Rheumatology, Department of Medical and Surgical Sciences, University of Padova, Italy; \*Microbiology and Immunology Unit, Pordenone, Italy*

**Introduction:** The fine antibody specificity towards protein components of uridine-enriched small nuclear ribonucleoproteins (UsnRNP) may be investigated by several methods including the Western immunoblot. Crucial in Western blot techniques' reliability is the origin and nature of the antigenic source.

**Aim:** To assess the significance of antibodies to B/B' proteins detected by Western immunoblot in connective tissue disease (CTD) patients.

**Methods:** Three hundred and forty-eight patients with well diagnosed CTD (101 SLE, 51 systemic sclerosis, 53 primary Sjogren's syndrome, 27 poly/dermatomyositis, 15 rheumatoid arthritis and 101 overlap CTD) and 31 matched healthy subjects were studied. In addition, sera from 13 patients with primary Epstein-Barr virus (EBV) infection (10 in acute primary infection and 3 with anamnestic past infection) and high titer IgG anti-EBV antibodies were tested. IgG anti-UsnRNP as well as anti-ribosomal P protein antibodies were determined by Western blotting on total Raji cell extract (a cell line transformed by EBV). Antinuclear and anti-dsDNA antibodies were detected by indirect immunofluorescence on HEP-2 cells and *Crithidia luciliae* respectively, anti-ENA by counterimmunoelectrophoresis. Statistical analysis was performed by chi-square test.

**Results:** An unsuspectedly high frequency of anti-B/B' antibodies was found, confined to SLE (54.4%) and overlap CTD with SLE

features (55.2%). Anti-B/B' antibodies were closely associated with other anti-UsnRNP antibodies ( $P < 0.0001$ ), gel precipitating anti-nRNP antibodies ( $P < 0.0001$ ) and anti-ribosomal P antibodies ( $P = 0.0013$ ). Band patterns unequivocally different from those obtained with autoimmune sera, were provided by anti-EBV positive sera. Noteworthy, a peptide with an apparent MW corresponding to that of B peptide (28kDa) was clearly recognized by 9/10 sera from active EBV infection but not by anamnestic EBV infection sera.

**Conclusions:** The Sm spliceosomal complex is one of the most important targeted autoantigens in SLE. Western immunoblot on Raji cells provides a reliably sensitive and specific antigenic source for anti-Sm B/B' antibodies. Such high immunoreactivity could be explained by the strong cross-reactive potential of B/B' proteins and not by EBV cell transformation. Further studies are in progress to comparatively evaluate the suitability of other cell lines as an antigenic source.

#### P70

#### Comparison of different methods for the detection of the fine specificity of anti-Ro/SSA response

I Cavazzana, F Franceschini, M Quinzanini, P Airò, A Brucato, R Cattaneo

*Clinical Immunology Unit and Chair, Spedali Civili and University of Brescia; Division of Medicine, Niguarda Hospital, Milan, Italy*

**Background:** the determination of the fine specificity of anti-Ro/SSA response is useful in the classification of the risk for the occurrence of congenital complete heart block (CCHB) in newborn of anti-Ro/SSA mothers.

**Aim of the study:** to evaluate different methods for the detection of anti-52 and 60 kD Ro/SSA antibodies.

**Patients and methods:** 132 sera (82 from anti-Ro/SSA patients by counterimmunoelectrophoresis (CIE), 30 from anti-ENA positive/anti-Ro/SSA negative and 20 from ANA and anti-ENA negative) were tested by ELISA with recombinant 52 and 60 kD Ro protein (Pharmacia Upjohn, Germany) and immunoblotting (IB) with human spleen extract (HSE) as substrate to the aim of determining the fine specificity of anti-Ro response. In addition, 21 sera from mothers of CCHB children were tested by CIE, two ELISAs with recombinant proteins (Pharmacia and Euro-diagnostica, The Netherlands), two IBs with HSE and with HEP-2 extract as substrates (MarDx, USA).

**Results:** the total agreement between ELISA (Pharmacia) and IB (HSE) was 76% for anti-Ro 60 kD and 44% for anti-Ro 52 kD. The ELISA was more sensitive than IB both for anti-Ro 60 kD (91% vs 84%) and for anti-Ro 52 kD detection (82% vs 51%). Seven sera positive by CIE were negative by IB (non blotters): six of these sera were positive for anti-60 kD and 2 for anti-52 kD by ELISA. The mean antibody titre for anti-60 kD was significantly lower ( $P < 0.00005$ ) than that of sera detected by IB.

A correlation ranging from 78 to 100% was detected between the different methods testing the sera from CCHB mothers. The agreement between the IB methods for anti-Ro 60 kD and for anti-52 kD was 79% and 68.5% respectively while between the ELISAs was 44% and 67% respectively. The best agreement obtained comparing IB and ELISA for anti-Ro 60 and 52 kD was 78% between IB with HSE and ELISA (Euro-diagnostica).

**Conclusions:** ELISA seems to be the most sensitive method to detect the fine specificity of anti-Ro/SSA response. The majority of IB negative/CIE positive sera (non blotters) were positive for anti-60 kD by ELISA at low titer. IB with HSE as substrate performed slightly better (p not significant) than IB with HEP-2 cells extract in CCHB mothers.



## P74

### Recombinant anti-P proteins antibodies isolated from human autoimmune library: reactivity, specificity and epitope recognition

S Zampieri, A Ghirardello, A Doria, WH van Venrooij\* and JMH Raats\*

Department of Medical and Surgical Sciences. Rheumatology Division. University of Padova, Italy; \*Department of Biochemistry. University of Nijmegen. Nijmegen, The Netherlands

**Introduction:** The ribosomal phosphoproteins P0, P1 and P2 are targeted by autoantibodies in SLE. The presence in the patient sera of the anti-P antibodies is highly specific for the disease and correlates with psychiatric, renal and liver involvement. In order to better characterize these autoantibodies (reactivity, specificity and epitope recognition), recombinant anti-P monoclonal antibodies were isolated from a human SLE patient derived phage display library.

**Methods:** Two synthetic peptides were used to select the recombinant anti-P antibody fragments: a synthetic peptide representing the C-22 common immunogenic region of the three P proteins and the multiple antigenic peptide (MAP) carrying four copies of the last 13 residues of the C-22. The human library was derived from the bone marrow lymphocytes of an anti-P positive SLE patient. The selected anti-P antibodies were tested for reactivity with the C-22, the MAP and a control panel of recombinant autoantigens in an ELISA assay. Specificity of the selected antibodies was further analyzed by immunoblotting and immunoprecipitation assays using Jurkat total cell extract. Indirect immunofluorescence staining on HEp-2 cells was also performed. Using different synthetic peptides derived from the C-22 peptide epitope recognition was further characterized in an ELISA assay. Sequencing of the selected antibody fragments was performed and the antibody sequence was compared to the nearest germ-line sequence. In all the experiments human anti-P positive control sera were included.

**Results:** Six recombinant anti-P antibodies were isolated from the human library when using the C-22 synthetic peptide. Some of the isolated antibodies reacted specifically with the C-22 antigen in ELISA, others recognized the ribosomal P proteins on Western blot, immunoprecipitated the P proteins from the Jurkat cell extract and showed cytoplasmic staining on HEp-2 cells in an immunofluorescence assay. The selected antibodies exhibited features similar to serum antibodies of the patients with respect to their reactivity, specificity and epitope recognition.

**Conclusions:** The phage display technology proves once again to be a very useful technique for the production of human monoclonal autoantibodies and for the characterization of the reactivity and specificity of these autoantibodies.

## P75

### Dominance of hydrophobic reading frames in complementarity determining region 3 of variable heavy chain genes from a patient with untreated SLE

B Yazdani-Biuki, R Brezinschek, T Dörner\*, J Hermann, H Mitterhammer, G Tilz, U Demel, T Müller, S Eder, J Gretler and HP Brezinschek

Department of Internal Medicine University Hospital Graz, Austria; \*Department of Rheumatology, University Hospital Charite Berlin, Germany

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of multiple autoantibodies (AAb), especially anti-dsDNA Ab. It is not known whether an aberrant V(D)J

recombination process itself predisposes to the generation of autoreactive Ab, or whether abnormalities in selection influences can lead to the generation of AAb. Immunoglobulin (Ig) heavy (H) and Ig light chains of an antibody are generated from variable (V), diversity (D) and joining (J) gene segments through V(D)J rearrangements. Diversification mechanisms inherent to the rearrangement reaction ensure that D elements can potentially be used in all reading frames (RF). In addition, D and J elements of the IgH chains encode the complementarity determining region (CDR) 3 that constitutes a significant part of the Ig antigen binding site. Since it has been suggested that the CDR3 of Ab in SLE is different from that found in normals, we compared the CDR3 obtained from Ab of an untreated SLE patient with that from normal individuals. D segments of Ab from normal donors are preferentially used in RF II (26/48, p\* 0.001) that most often encodes hydrophilic antibodies. Comparison of productive and nonproductive rearrangements suggests, that this is the result of the recombinational process rather than selection. In contrast, RF II was significantly less often used in SLE Ab (4/17, p\* 0.03). In both, normal and SLE Ab, D segments were significantly less often found utilizing RF that encode stop codons. Similar to the usage of RF II, in normals this seems to be the result of the recombination process rather than selection. Because of the low number of nonproductive rearrangements in the SLE analysis it is not possible to estimate whether this results from selection or the recombination process. In contrast to the analysis of the RF, no significant difference between the length or composition of the CDR3 from SLE and normal Ab was found.

## P76

### Comparative analysis of anti-histone and anti-chromatin antibody specificity in lupus erythematosus cell-positive and -negative sera and their relation to disease activity

G Schett\*, RL Rubin†, G Steiner\*, H Hiesberger\*, S Muller‡ and JS Smolen\*

\*Division of Rheumatology, General Hospital Vienna, Austria; †The Scripps Research Institute, La Jolla, California, USA; ‡Institut de Biologie Moleculaire & Cellulaire, Strasbourg, France

Anti-histone and anti-chromatin antibody responses play a central role in the autoimmune response of systemic lupus erythematosus (SLE). Furthermore, anti-histone H1 antibodies are essential for the formation of the lupus erythematosus cell (LEC) phenomenon. In this study, the binding properties of LEC+ and LEC- SLE sera to chromatin-associated nuclear antigens (histones H1, H2A, H2B, H3, H4; complexes of H2A-H2B, [H2A-H2B]-DNA, H1-DNA; total and H1-stripped chromatin; native and denatured DNA) were investigated. In addition, sera from patients with drug-induced lupus (by procainamide, hydralazine, or quinidine), as well as from patients with rheumatoid arthritis and osteoarthritis, were assessed. Enzyme-linked immunosorbent assay was used to detect specific antibody binding. Mirroring the important role of histone H1 in the formation of LE cells, anti-histone H1 reactivity was 8-fold higher in LEC+ sera than in LEC- sera. In addition, reactivities to most of the other antigens tested, i.e., other histones and histone-DNA complexes as well as chromatin and DNA, were significantly higher in LEC+ sera than in LEC- sera. All but 1 serum sample from the patients with drug-induced lupus were negative for LE cell formation as well as for anti-histone H1 reactivity, but displayed high antibody reactivities to histone-DNA complexes, including chromatin. Sera from patients with rheumatoid arthritis and osteoarthritis did not show significant binding to these antigens. When comparing the clinical features of LEC+ and LEC- SLE patients, severe organ involvement, including nephritis and



**P80**

**Improving an anti-β<sub>2</sub>GPI ELISA by reducing the influence of a blocking agent**

**A Ambrožič, B Božič, M Hojnik and T Kveder**

*Department of Rheumatology, University Medical Centre, Ljubljana, Slovenia*

There are still considerable interlaboratory differences in positivity rate in anti-β<sub>2</sub>GPI ELISA. We have already shown that BSA as a blocking agent could introduce a substantial interference effect in an anti-β<sub>2</sub>GPI ELISA.

The aim of this study was to validate and possibly reduce an interference effect of different blocking agents on the detection of IgG anti-β<sub>2</sub>GPI antibodies by ELISA.

We used Costar high binding plates coated with affinity purified human β<sub>2</sub>GPI and blocked with 1% BSA or 3% gelatin in PBS. Selected sera (20 NHS, 20 APS sera and 10 sera from children with atopic diseases) were diluted in PBS containing 0.05% Tween (PBS-T) or in 0.1% BSA/PBS-T or in 1% gelatin/PBS-T.

When plates were blocked with BSA and samples diluted in PBS-T, 11/50 sera expressed values above the cut-off level in the wells coated with β<sub>2</sub>GPI and also substantial binding in sample blanks wells (SB) mostly exceeding the binding to the antigen, therefore these samples were considered negative (average SB for all sera =  $x \pm SD = 63 \pm 127$  mOD). The specificity of IgG antibodies yielding high background bindings was confirmed by direct binding to BSA on solid phase (correlation with SB:  $P < 0.001$ ,  $R^2 = 0.88$ ) and efficient inhibition by fluid phase BSA. Further, the sera were diluted in 0.1% BSA/PBS-T, which resulted in negligible binding to BSA either directly coated on the plates or used as the blocking agent and hence lowered SB to insignificant levels (SB =  $13 \pm 9$  mOD). Following this modification, 3/11 sera previously found negative due to high SB values, clearly expressed low positive IgG anti-β<sub>2</sub>GPI values. The inhibition of anti-BSA with 0.1% BSA in fluid phase was almost complete in 3 minutes, suggesting that longer preincubation time may be unnecessary.

1% gelatin/PBS-T as the sample diluent buffer did not prevent the substantial binding to BSA used as the blocking agent either (SB for 20 sera with the highest binding to BSA =  $203 \pm 262$  mOD). The same was true even when the plates were blocked with 3% gelatin and samples diluted either in PBS-T (SB =  $117 \pm 120$  mOD) or in 0.1% BSA/PBS-T (SB =  $127 \pm 122$  mOD) generating substantial SB values in 18/38 tested sera. Similarly to BSA, significantly lower background bindings were reached only when gelatin was used as the blocking agent and 1% gelatin added to the sample diluent buffer (SB =  $30 \pm 21$  mOD).

To reduce the interference effects of a blocking agent it was essential to dilute sera in a buffer containing the same agent. Since the binding to BSA or gelatin was detected in both normal human and patients' sera we suggest to follow this general guideline in anti-β<sub>2</sub>GPI ELISA to better define the cut-off points and to more accurately verify not only high, but also most of low positive results.

**P81**

**Heterogeneous behaviour of anti-β<sub>2</sub>-glycoprotein I antibodies on different "high binding" microtiter plates**

**A Ambrožič\*, T Kveder\*, K Ichikawa‡, T Avčin†, M Hojnik\*, B Božič\*, T Koike\***

*\*Department of Rheumatology and †Department of Paediatrics, University Medical Centre, Ljubljana, Slovenia. ‡Department of Medicine II, Hokkaido University School of Medicine, Sapporo, Japan*

We recently identified anti-β<sub>2</sub>GPI antibodies in a high proportion of sera from children with atopic dermatitis (AD) and showed that

these anti-β<sub>2</sub>GPI most probably recognise domain V of β<sub>2</sub>GPI by contrast to anti-β<sub>2</sub>GPI from patients with the anti-phospholipid syndrome (APS) which epitopes apparently reside in domain I or IV.

The aim of the present study was to compare the binding of IgG anti-β<sub>2</sub>GPI in AD and APS on four representative commercially available types of high binding microtiter plates.

Selected plates: Costar, Nunc, Linbro and Sumilon C. Randomly selected sera from 29 children with AD and sera from 43 SLE patients (24 with secondary APS) were tested by anti-β<sub>2</sub>GPI ELISA using affinity purified β<sub>2</sub>GPI. Assays were calibrated with the HCAL, chimeric anti-β<sub>2</sub>GPI monoclonal antibodies with human γ1 constant regions.

The calibration curves for HCAL were practically the same on all four types of plates. Sera from 7/24 APS patients with medium or high anti-β<sub>2</sub>GPI levels showed similar binding properties on all four plates, while 3/24 sera expressed values either slightly above or below the cut-off points. On the other hand, anti-β<sub>2</sub>GPI from AD sera showed very similar binding on Costar, Nunc and Linbro plates, while only 3/13 positive sera with the highest values on these 3 types of plates expressed low positive values for IgG anti-β<sub>2</sub>GPI on Sumilon C plates (Table 1). Except for one serum (low positive on Linbro plate) all sera from SLE patients without APS were negative on all the plates.

Our results point to substantial differences in the binding to β<sub>2</sub>GPI coated on different microtiter plates by anti-β<sub>2</sub>GPI in AD (with no signs of APS) but not by anti-β<sub>2</sub>GPI in APS. In contrast to the other plate types, Sumilon C plates coated with β<sub>2</sub>GPI bound only minimally antibodies from AD children. If such anti-β<sub>2</sub>GPI prove non-thrombogenic, we will be able to increase the specificity of detecting anti-β<sub>2</sub>GPI relevant for APS by the use of this type of microtiter plates. Alternatively, if both anti-β<sub>2</sub>GPI specificities prove thrombogenic, we will be able to increase the sensitivity of the assay system by the use of other less discriminatory types of plates.

Table 1 COSTAR NUNC LINBRO SUMILON C

	k		R <sup>2</sup>		N		k		R <sup>2</sup>		N		k		R <sup>2</sup>		N	
APS (n = 24)	1.00	1.00	8	1.06	0.99	8	1.09	0.95	9	0.84	0.99	9						
AD (n = 29)	1.00	1.00	13	1.05	0.97	13	0.87	0.92	13	0.22*	0.63	3*						

k, slope of linear regression plot and R<sup>2</sup> - determination coefficient: both compared to Costar. N, number of IgG anti-β<sub>2</sub>GPI positive sera in the group. \*P < 0.001 (significant difference when compared with Costar, Nunc or Linbro)

**P82**

**Oxidation of β<sub>2</sub>-glycoprotein I (β<sub>2</sub>GPI) by the hydroxyl radical alters phospholipid binding and modulates recognition by anti-β<sub>2</sub>GPI autoantibodies**

**J Arvieux, V Regnault, E Hachulla, L Darnige, F Berthou and P Youinou**

*Laboratoire d'Immunologie, Institut de Synergie des Sciences et de la Santé, CHU Brest, France*

We investigated whether β<sub>2</sub>GPI, the key antigen in the antiphospholipid syndrome, is susceptible to oxidative modifications by the hydroxyl radical (°OH) that may influence its lipid-binding and antigenic properties. We compared the effects on human and bovine β<sub>2</sub>GPI of °OH free radicals generated by γ-radiolysis of water with <sup>137</sup>Cs and by the Fenton system composed of Fe-EDTA, ascorbate and H<sub>2</sub>O<sub>2</sub>. Radiolytic °OH caused a dose-dependent loss of tryptophan, production of di-tyrosine and carbonyl groups, dimerization



**Materials:** Twenty women with SO and 19 with AO FM, matched as to age and clinical symptoms, were studied for a multitude of antimicrobial and autoantibodies in serum. Markers of inflammation, immune activation and nerve cell damage were looked for in CSF and serum. All patients had longstanding disease.

**Results:** More patients with AO FM had IgM antibodies to enteroviruses, but PCR amplification showed no signs of enteroviral genome in CSF. All other antimicrobial and autoantibodies were similar in the two groups. However, in the SO FM patients we found strongly increased intrathecal IgA production as shown by extended indices but normal albumin ratio indicating normal blood/CSF barrier function. Intrathecal IgM production was increased in a few SO FM patients but IgG production was normal in all FM patients. Myelin basic protein (MBP) levels were normal in CSF of AO FM patients but very low in the SO patient group.

**Conclusions and discussion:** In FM characterized by an insidious onset of symptoms an immunoinflammatory mechanism involving IgA production in the brain may be a driving pathogenetic mechanism. Patients having experienced an acute onset of FM after a flue-like episode are likely to suffer from sequelae after earlier encephalitis, showing no signs of immune activation. The abnormally low MBP levels in the CSF of SO FM patients are yet unexplained. Our findings strongly support the concept that FM is a result of brain abnormalities that lead to disordered sensory processing and widespread allodynia.

## P86

### Disorders of the system of hemostasis and biochemical parameters of NZB/NZW F1 mice

AV Arshinov\*, OA Nazarova†, GN Pleskovskaya‡ and VV Redko\*

\*Medical Academic Yaroslavl; †Medical Academy, Ivanovo; ‡Institute of Rheumatology, Moscow, Russia

Object of a research. A research of interaction of coagulation and biochemical parameters of NZB/NZW F1 mice with spontaneous explicating lupus like disease.

**Methods:** 120 female mice of a line NZB/NZW F1 3 months age were investigated. Coagulation tests were used: counting platelets, activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), concentration of fibrinogen, soluble fibrin monomer complexes (SFMC); parameters of platelet aggregate (spontaneous and induced with ristocetin, collagen and ADF). Biochemical parameters of serotonin and cortisol were investigated. An electronic microscopy of microvessels was investigated also.

**Results:** Significant (more than twice) decreasing the amount of platelets of NZB/NZW F1 mice, elongation of parameters of the coagulation tests (APTT  $40,0 \pm 2,7$  sec, control  $27,6 \pm 2,5$  sec) ( $P < 0,01$ ), decreasing the concentration of fibrinogen ( $1,1 \pm 0,2$  g/l, control  $5,2 \pm 0,6$  g/l), increasing the level SFMC ( $28,1 \times 10^{-2} \pm 1,6$  g/l, control  $8,9 \times 10^{-2} \pm 1,03$  g/l), increasing the parameter of spontaneous platelets aggregate ( $20,3 \pm 1,96$  %, control  $2,5 \pm 0,6$  %) and aggregate of platelets with ADF ( $12,8 \pm 1,3$  %, control  $9,0 \pm 0,8$  %) decreasing the aggregate with collagen ( $4,4 \pm 0,6$  %, control  $9,3 \pm 0,8$  %) were registered. The concentration of "plasma" serotonin was increased ( $0,065$  mcg/ml, control  $0,042$  mcg/ml), the level of cortisol was considerably reduced ( $0,4 \pm 0,09$  mcg/ml, control  $1,03$  mcg/ml). The correlation between increasing the concentration of "plasma" serotonin, increasing the parameter of the spontaneous aggregate of platelets, increasing the concentration of SFMC, elongation of the coagulation tests and decreasing the concentration of "platelet" serotonin were marked. By the electronic microscopy the dystrophy of endothelium is registered.

**Conclusion:** Thus the endothelial damage of NZB/NZW F1 mice was accompanied by the expressed activation of a system of hemostasis, amplifying the aggregate of platelets and increasing the

release of serotonin from them. At the same time the significant decreasing the concentration of cortisol was found. These disorders of hemostasis are typical for DIC syndrome. Therefore, it is possible to use NZB/NZW F1 mice as an animal model for study of disorders of hemostasis, including DIC syndrome, for the patients with SLE.

## Poster Discussion F

### Innovative Therapies

## P87

### Adenoviral gene transfer of tissue inhibitors of metalloproteinases (TIMPs) reduces the invasive behaviour of rheumatoid fibroblast-like synoviocytes

WH Van der Laan\*\*†, L Huisman\*, E Pieterman†, PHA Quax\*, JM TeKoppele\*, FC Breedveld†, JH Verheijen\* and TWJ Huizinga†

\*Division of Vascular and Connective Tissue Research, TNO Prevention and Health, Leiden; †Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

In rheumatoid arthritis (RA), an excess of proteolytic enzymes secreted at the synovium-cartilage junction results in the invasion of the articular cartilage by synovial cells. The aims of the present study were to investigate the effects of overexpression of TIMP-1 and TIMP-3 on: 1) the invasive behaviour of rheumatoid fibroblast-like synoviocytes and 2) cell proliferation and apoptosis.

The day before the experiments, the synoviocytes were infected with adenoviral vectors encoding TIMP-1 or TIMP-3 or with a control vector (AdLacZ). A Transwell system was used to study invasion of the cells. After 3 days, the invaded cells were counted using a microscope. Proliferation was assessed by measuring 3H-thymidine incorporation and cell counting. Apoptosis was assessed at 1-4 days after transduction using an Annexin V-FITC kit.

Both TIMP-1 and TIMP-3 overexpression resulted in a significant reduction, respectively 60% ( $P < 0.001$ ) and 80% ( $P < 0.001$ ), of invasiveness of the synoviocytes as compared to the AdLacZ-transduced cells. In all cases, TIMP-3 was superior to TIMP-1 ( $P = 0.02$ ). Cell proliferation was significantly reduced by TIMP-3 overexpression (40%;  $P < 0.05$ ) and to a lesser extent by TIMP-1 (20%;  $P < 0.05$ ) as compared to AdLacZ. There were little differences in % of apoptotic cells between the non-transduced, AdTIMP-1, AdTIMP-3 or AdLacZ transduced cells up to 4 days after transduction. A maximum of 15% of the AdTIMP-3 transduced cells were in apoptosis as compared to a maximum of 12% in the other conditions. These results show that the invasive behavior of RA-FLSs can be strongly inhibited by overexpression of TIMPs. Both MMP inhibition and a reduction of proliferation appear to contribute to this effect. The superior effect of TIMP-3 may be due to a stronger effect on proliferation or to differences in the inhibitory profile of TIMP-1 and TIMP-3. To limit joint destruction in rheumatoid arthritis, inhibition of cartilage invasion by the pannus tissue by TIMP overexpression may be a useful approach.



**P91**

**Development of a doxycycline inducible AAV vector for long term *in vivo* viral IL-10 gene transfer in rheumatoid arthritis**

**F Apparailly\***, **D Noël\***, **V Millet\***, **C Jacquet\***, **J Sany\*\*** and **C Jorgensen\*\***

\*INSERM U475, †Immunorhumatologie, CHU Lapeyronie, Montpellier, France

**Objectives.** The recent development of AAV vectors (adeno-associated virus) offers new perspectives for cytokine gene transfer in RA as they are non pathogenic and allow long term transgene expression *in vivo*. Moreover, we propose to regulate vIL-10 expression with tetracycline derivative (tetON system). The purpose of this study was to assess the potential long-term gene expression regulation of a recombinant AAV vector expressing vIL-10 in human rheumatoid synovial tissue and its efficiency in collagen-induced arthritis (CIA).

**Methods.** The AAV-tetON-vIL10 vector contains two transcriptional units oriented in opposite directions, with a central bi-directional SV40 polyA. Sequences encoding the transcriptional activator rTA, which confers doxycycline transgene induction, is inserted downstream a retroviral LTR promoter. A minimal human CMV promoter, flanked with tetracycline operator motifs, controls the transcription of vIL-10. Human RA synoviocytes were infected *in vitro* with AAV-tetON-vIL10 (500 MOI) and vIL-10 secretion was assessed by ELISA after addition of 5 µg/ml doxycycline (dox). Therapeutic efficiency of the vector was achieved after intra-muscular injection (1.5 x 10<sup>9</sup> pi) in DBA1 mice with CIA in the presence of doxycycline in the drinking water (0.2 mg/ml).

**Results.** Viral IL-10 secretion by RA synoviocytes was increased 40-fold in presence of dox (233 ng/ml/10<sup>6</sup> cells) and returned to basal level 24 hr after dox removal. In CIA, serum vIL-10 increased to 0.38 ng/ml, 5 weeks after gene transfer in animals under diet dox. RT-PCR analysis showed vIL-10 transcription in the muscle up to 14 weeks, without diffusion in other organs. We observed a decrease of CIA incidence (30% versus 89% in AAV-GFP injected control group) and of paw swelling (1.68±0.04 versus 1.81±0.15 on day 35 post-immunization, *P* < 0.0003).

**Conclusions.** AAV vectors conferred safe and inducible long-term expression of vIL-10. These data support AAV-tetON-vIL10 as a therapeutical tool for gene therapy in RA.

**P92**

**IL-18 blockade is a potential disease-modifying therapy for rheumatoid arthritis**

**C Plater-Zyberk<sup>§</sup>**, **LAB Joosten\***, **MMA Helsen\***, **P Sattounet-Roche<sup>§</sup>**, **C Siegfried<sup>§</sup>**, **S Alouani<sup>§</sup>**, **FAJ van de Loo\***, **P Graber<sup>§</sup>**, **S Aloni†**, **CA Dinarello‡**, **WB van den Berg\*** and **Y Chvatchko<sup>§</sup>**

<sup>§</sup>Serono Pharmaceutical Research Institute, 14 chemin des Aulx, 1228 Geneva, Switzerland; \*Rheumatology Research Laboratory, University Medical Center St-Radboud, Nijmegen, The Netherlands; †InterPharma Laboratories, Nes Ziona, Israel; ‡Department of Medicine, Division of Infectious Diseases, University of Colorado Health Sciences, Denver, Colorado, USA

**Introduction:** Interleukin-18 (IL-18) has been demonstrated as promoting the development of a TH1 response *in vivo* in synergy with IL-12. Significant levels of IL-18 and IL-12 have been detected in the joints of patients with rheumatoid arthritis (RA).

**Aim:** To define the therapeutic potentials of IL-18 blockade in RA by investigating the effect of neutralising endogenous IL-18 in the experimental CIA mouse model.

**Methods:** Two distinct IL-18 neutralising strategies, i.e., a recombinant human IL-18 binding protein (hIL-18BP) and a polyclonal anti-IL-

18 IgG, were used to treat CIA mice in a therapeutic protocol (after disease onset). The effect on disease severity (visual scores) as well as parameters of cartilage and bone destruction were evaluated.

**Results:** Clinical scores were significantly reduced after IL-18 blockade (rhIL-18BP 1 mg/kg, *P* < 0.001, *n* = 13; rhIL-18BP 0.25 mg/kg, *P* < 0.05, *n* = 7; anti-IL18 IgG, 2 mg, *P* < 0.05, *n* = 9, Mann Whitney test, treated versus placebo groups). Histological examination showed cartilage protection (decrease erosion scores, *P* < 0.05) that was accompanied by significantly reduced levels of serum cartilage oligomeric matrix protein (an indicator of cartilage turnover) and VDIPEN expression (a neoepitope present after digestion by matrix metalloproteinases). X-ray analysis of joints provided evidence of reduced bone erosion. Serum IL-6 levels were diminished in the treated animals.

**Conclusions:** These results clearly demonstrate that blocking endogenous IL-18 is therapeutically efficacious in the CIA model and support the use of IL-18 neutralisation as a novel cartilage and bone protective therapy for the treatment of destructive arthritis. Recombinant hIL-18BP could therefore represent a new disease-modifying anti-rheumatic drug that warrants testing in clinical trials in patients with rheumatoid arthritis.

**P93**

**Digital vasculitis in a patient with rheumatoid arthritis: good response on anti-TNF blockade**

**F van den Hoogen, A den Broeder, M Zandbelt and L van de Putte**

Department of Rheumatology, University Medical Center St. Radboud, Nijmegen, The Netherlands

Rheumatoid arthritis (RA) may be complicated by vasculitis. Vasculitis usually affects small vessels of the skin causing nailfold infarcts, but may also affect larger vessels and cause severe damage to internal organs. In such cases, treatment with high doses of corticosteroids or other immunosuppressive drugs may be necessary. TNF-alpha blockade has been shown to be an effective and safe treatment for RA, but thus far no reports have addressed the effect of TNF-alpha blockade on extra-articular manifestations of RA, such as vasculitis. We report a patient with RA and nailfold infarcts which repeatedly disappeared for several weeks following monthly *i.v.* injections with an anti-TNF alpha receptor fusion protein.

A 46 year old woman was diagnosed as having rheumatoid factor positive, erosive RA in 1982. Due to the uncontrollable disease she was included in 1994 in a study with Ro 45-2081, a fusion protein combining two p55 TNF receptors with the Fc component of an IgG human antibody (Roche, Basel, Switzerland, sTNFR:Fc). After a three months placebo controlled phase she was treated with 50mg sTNFR:Fc every four weeks. Clinical response was impressive with swollen joint counts decreasing from 32 to 5 and C-reactive protein CRP levels declining from 95 at baseline to 20 after the first injection. Low disease activity was sustained for the following years. Besides sTNFR:Fc her medication consisted of oral prednisone 5 mg a day and occasionally paracetamol 500 mg. In the spring of 1999 she first noticed nailfold infarcts on the fingers of both hands. These lesions disappeared after every injection of sTNFR:Fc and reappeared three weeks thereafter when the clinical effects of sTNFR:Fc were decreasing. This effect on the digital vasculitis has been well documented during several cycles of sTNFR:Fc administration.

**Conclusion:** The prompt disappearance of nailfold infarcts after sTNFR:Fc administration observed in our patient strongly suggests a therapeutic effect of sTNFR:Fc on active vasculitis. This observation raises the question whether blocking of TNF-alpha might also be effective in more severe forms of vasculitis and possibly other extra-articular manifestations of RA, some of which are life threatening and are currently treated with high doses of corticosteroids and immunosuppressive drugs.



**P97**

**Newer immunomodulating drugs in rheumatoid arthritis may precipitate glomerulonephritis**

**H Nielsen, E Kemp, LJ Petersen, AN Gam, J Dahlager, T Horn, S Larsen and S Olsen**

*Department of Rheumatology/Nephrology/Pathology, Herlev and Glostrup University Hospitals of Copenhagen and Department of Internal Medicine, Roskilde Hospital, Denmark*

Three patients with rheumatoid arthritis on newer immunomodulating therapy, developed acute glomerulonephritis. Two of the patients were treated with tumour necrosis factor blockade ( Etanercept, 25 mg sc. twice a week) and one with leflunomide (Arava, 20mg daily) in addition to the conventional medical treatment. All of the patients developed unexpected blood and urinary abnormalities, two of them after treatment with Etanercept for eleven- and one month respectively. Renal biopsies showed in the patients with long term Etanercept treatment, focal proliferative glomerulonephritis with cellular crescents in 30% of all glomerular sections. The biopsy showed mesangial deposits of IgA. This patient was suspected clinically for subacute bacterial endocarditis, however all data were negative. In the other patient treated with Etanercept for only four weeks slightly diffuse mesangial proliferative glomerulonephritis was demonstrated. Electron microscopy of this biopsy showed distinct mesangial matrix changes "moth-eaten" appearance. In the patient treated with Arava during four weeks, biopsy showed focal proliferative glomerulonephritis with cellular crescents in 7% of all glomerular sections and with IgA mesangial deposits.

Two of the patients thus had IgA glomerulonephritis. The diagnosis of the third one was inconclusive, as regard the present of IgA, but pathology could represent IgA glomerulonephritis in resolution.

The relation in time of sign of renal disease to the treatment with Etanercept and Arava makes it probable that renal disease was related to these drugs. It is generally assumed that IgA glomerulonephritis is caused by the deposition of immune complexes, but details of antigen(s) are in these cases unknown. We finally regard it as a possibility that the immunomodulation caused by these new drugs may facilitate silent infection and subsequently development of IgA glomerulonephritis. At least in long term treated patients this aetiology could not be excluded.

**P98**

**The use of Ribomunyl® in the immunomodulatory treatment of rats with adjuvant arthritis**

**J Rovenský, K Svík and M Stanciková**

*Research Institute of Rheumatic Diseases, Piešťany, Slovakia*

Immunomodulatory therapy of inflammatory rheumatic diseases, especially in refractory forms of systemic lupus erythematosus (Rovensky *et al.*) and rheumatoid arthritis (Mateicka *et al.*) has its tradition. The very immunosuppressive therapy may induce the development of resistance and to participate in recurrent secondary infections in patients suffering from systemic diseases of the connective tissue. Alternatives of immunomodulatory therapy are therefore sought for that would eliminate some adverse effects of immunosuppressive agents on the cell-mediated and non-specific immunity function, and would favorably affect the clinical condition of the patient.

To verify our working hypothesis concerning the appropriateness of immunomodulatory therapy with Ribomunyl®, the adjuvant arthritis model in rats was chosen. Following drugs and their combinations were orally administered to animals in a long-term prophylactic course: Cyclosporine A (CyA, 2,5 mg/kg/day), methotrexate (MTX, 0,3 mg/kg, 2 times a week), Ribomunyl® (25 mg/kg 4 times a

week), CyA+MTX, CyA+Ribomunyl®, MTX+Ribomunyl®, and the three-combination of CyA+MTX+ Ribomunyl®. When given in combination, both the doses and the frequency of administration were the same as when the drugs were administered alone. The following markers of inflammation and arthritic process were measured: serum albumin, joint X-ray, hind paw swelling, and on day 40 of the study, bone mineral density (BMD) and bone mineral content (BMC).

Our results showed that Ribomunyl® alone has no marked effect on markers of inflammation and arthritis in animals with adjuvant arthritis. When combined with the immunosuppressive drugs CyA and MTX, a similar and/or better therapeutical effect was observed than with the basic drug without Ribomunyl®. However, the effect of the three-combination of CyA+MTX+Ribomunyl® was rather remarkable. This combination had the most pronounced therapeutical effect on rats with adjuvant arthritis. It significantly inhibited inflammatory and arthritic markers as well as BMD and BMC reductions.

Our results obtained using the adjuvant arthritis model suggest that immunomodulatory procedures are promising. These results therefore need to be verified in additional animal models and markers of cell-mediated immunity and/or cytokines involved in the induction of this therapeutic effect should be investigated.

Rovensky J. *et al.*: Levamisole treatment of systemic lupus erythematosus. *Arthritis Rheum* 1982; 24: 470-471.

Mateicka *et al.*: Immunomodulatory treatment with Biostim (Roussel Uclaf) in patients with rheumatoid arthritis (Preliminary follow-up of group with 10 patients). *Rheumatologia* 1992;6: 129-133.

**P99**

**Thymosin beta4 sulphoxide: potential role in resolution of inflammation?**

**JD Young, JA Gracie, RD Stevenson, AJ Lawrence, FY Liew\* and IB McInnes**

*Centre for Rheumatic Diseases, University Department of Medicine, Royal Infirmary, Glasgow, G31 2ER and \*Department of Immunology, University of Glasgow, Glasgow, G11 6NT, UK*

**Background:** Thymosin beta 4 sulphoxide (Tb4so) has previously been shown to be produced by glucocorticoid-treated monocytes (1). This highly conserved intracellular peptide possesses 'moonlighting' functions in the modulation of inflammatory responses, and may represent a natural down-regulator of inflammation *in vivo*. We have investigated the mechanisms of action of Tb4so primarily on neutrophils by studying its effects on *in vitro* and *in vivo* models.

**Methods:** Effect of Tb4so on assays of neutrophil function included chemotaxis and respiratory burst. Apoptosis was measured as Annexin-V/PI binding by FACS and macrophages were stained for phagocytic uptake of apoptotic neutrophils by the presence of neutrophil-specific myeloperoxidase. *In vivo*, the effect of administration of Tb4so on carrageenan-induced inflammation was explored.

**Results:** Tb4so significantly inhibited fMLP-induced chemotaxis ( $P < 0.005$ ) and respiratory burst of human neutrophils in a dose dependent manner (100% vs 23%,  $P < 0.05$ ). Further, it increased the rate of apoptosis in neutrophils ( $20.5 \pm 1.9\%$ ,  $P < 0.05$ ) and their subsequent phagocytic uptake by macrophages. *In vivo*, Tb4so was a potent inhibitor of neutrophil mediated carrageenan-induced inflammation in BALB/c mice (1.2mm vs 0.6mm  $P < 0.001$  at 24h).

**Conclusions:** Tb4so is an anti-inflammatory peptide that down-regulates neutrophil mediated inflammation. The mechanism of action appears to be, at least in part, via induction of neutrophil apoptosis and their clearance by phagocytic macrophages. These results suggest therapeutic potential for Tb4so.

1. Young *et al* 1999 *Nature Med.* 5:1424



-SE/SE or SE/XP4p genotypes are associated with a high risk to develop RA.  
 -XP4p/XP4p or SE/XP4n genotypes are neutral  
 -XP4p/XP4p or XP4n/XP4n genotypes are protective with respect to the development of RA.

**P104**

**Positivity of HLA-DRB1 rheumatoid epitope does not predict the course of juvenile idiopathic arthritis in Czech children**

**O Cinek, P Vavrinová, P Drevínek, M Suková, Š Rádová\* and J Vavrinec**

*2nd Paediatric Department, 2nd Medical Faculty, \*University Hospital Motol, Prague, Czech Republic*

**Aims:** Association of juvenile idiopathic arthritis (JIA) with HLA class II still remains unresolved. Our study investigated whether presence of the HLA-DRB1 amino acid 70-74 rheumatoid epitope (RE) is a predictive factor of the disease course.

**Patients and methods:** We analysed 74 consecutive patients with JIA diagnosed and classified according to ILAR criteria, aged 11.2 ± 4.2 (mean ± SD), 35 boys and 39 girls. The numbers of children having oligoarticular, polyarticular, and systemic form of JIA were 24, 40, and 10, respectively. HLA-DRB1 alleles carrying the QRRAA, QKRAA, and RRRAA motifs of RE were typed for using PCR with sequence-specific primers.

**Results and Conclusions:** There were no significant differences in frequency of the RE, or its particular motifs, among the three forms of JIA.

	JIA oligo.	JIA poly.	JIA systemic	Total	P value
RE negative	16	25	6	47	N.S.
RE positive	8	15	4	27	N.S.
Total	24	40	10	74	

The 2x2 table were tested using chi2 test with Yate's correction, or Fisher exact test where appropriate.

We therefore conclude that simple positivity of the DRB1 rheumatoid epitope is not a likely predictive factor of the JIA course in Czech children.

**P105**

**In Marseille, Southern France, HLA-B2702 carries higher risk than HLA-B2705 to develop ankylosing spondylitis (AS)**

**S Guis, W Nielsen, G Boetsch, O Dutour, P Mercier, D Reviron and J Roudier**

*Rheumatology Ward La Conception, INSERM EMI9940, EFS Alpes Provence Histocompatibility, Anthropology UMR6578, Marseille, France*

HLA-B27 subtypes were defined by molecular typing in 45 patients with AS and 90 controls from Marseille. We subdivided patients and controls in 2 subgroups, according to the birth place of their grandparents: 19 patients and 38 controls constituted the Spanish/North African subgroup; 26 patients and 52 controls constituted the "French" subgroup. In patients from the Spanish/North African subgroup, the frequency of B2702 was higher (74%) than in controls from the same area (21%)*pc*<0.01 and the frequency of B2705 was lower in patients (25%) than in controls (79%)*pc*<0.01. In patients from the French group, the frequency of B2702 was higher (7%) than in controls (2%)(N.S.).

Thus, in the population of Southern France, B2702 seems to carry higher risk to develop AS than B2705.

**P106**

**Juvenile idiopathic arthritis is associated to a functionally active polymorphism in the SH2D2A gene**

**A Smerdel, K-Z Dai, B Flato, R Ploski\*, O Forre and A Spurkland**

*IMMI, The National Hospital, Songsvannsveien 20,0027 Oslo, Norway; \*Institute of Rheumatology, Warsaw, Poland*

**Objective:** T cell specific adapter protein (TSA<sub>d</sub>) is involved in the negative control of T cell activation. The SH2D2A gene encoding TSA<sub>d</sub> is located on chromosome 1q21 which has been implicated in susceptibility to experimental autoimmune disorders in the mouse (chronic allergic encephalomyelitis and collagen-induced arthritis). Recently we found that short alleles of the SH2D2A gene promoter are associated with multiple sclerosis (MS). The aim of our study was to investigate whether the SH2D2A promoter polymorphism contributes to the genetic susceptibility to develop juvenile idiopathic arthritis (JIA).

**Methods:** DNA from 212 Norwegian patients with juvenile arthritis (categorized as systemic (*n* = 18), poly- RF+ (*n* = 12), poly- RF- (*n* = 61), oligo- (*n* = 87) and extended oligoarthritis (*n* = 31)) and 279 healthy unrelated Norwegian controls were genotyped for a functional GA repeat polymorphism in the promoter region of SH2D2A gene using an ABI automatic sequencing machine (ABI Prism™ XL377)

**Results:** The frequencies of the two shortest alleles GA13 and GA16 were increased among the JIA patients compared to the control; the GA13 significantly so (0.098 vs 0.053, OR=1,97, *P* = 0,0063). When we divided patients into subgroups only in the RF-positive polyarthritis group of patients there was no increases of any of the short alleles. All other subgroups of JIA showed an increased frequency of GA13, however only in the patients with oligoarthritis the increased frequency of GA13 allele reached significance (0.103, *P* = 0.017). When we analyzed the frequency of short alleles in relation to the occurrence of chronic iridocyclitis (CIC) in the group of patients with oligoarthritis (*n* = 14), we found that 57% of these patients carry at least one short allele compared to 46% of the patients without CIC (*n* = 73).

**Conclusion:** Our data indicate that the short alleles of the SH2D2A promoter associated with JIA patients could contribute to the genetic susceptibility of JIA, similar what we have observed in MS. It is possible that the short allele is a marker for particular clinical presentations. This we will investigate in further details.

**P107**

**Analysis of VH and VL mRNA in single synovial and peripheral B/plasma cells of patients with rheumatoid arthritis**

**S Ruzickova\*‡, J Vencovsky\*, O Krystufkova\*, Z Cimburek\*, J Sinkora†, O Horvath\* and T Doerner§**

*\*Institute of Rheumatology and Laboratory of Gene Expression, †Division of Immunology and Gnotobiology, ‡Department of Immunology, Institute of Microbiology CAS, Prague, Czech Republic; §Department of Rheumatology and Clinical Immunology, Charite University Hospital, Berlin, Germany*

**Introduction:** Synovial tissue in rheumatoid arthritis displays a complex infiltration of many cell types like T and B lymphocytes, plasma cells, follicular dendritic cells, macrophages etc. Presence of B and plasma cells results in secretion of large amounts of multiple pathologic autoantibodies.



**P110**

**Time to lupus nephritis: impact of gender and ethnicity**

**VA Seligman, H Li, JL Olson and LA Criswell**

*Department of Medicine, UCSF, San Francisco, CA 94143-0633; Department of Human Genetics, UCD, Davis, CA 95616, USA*

**Objective:** There is a paucity of literature regarding the time to development of nephritis among SLE patients. Our goal was to define this important parameter for a large multi-ethnic cohort, with an emphasis on the impact of gender and ethnicity.

**Methods:** 779 SLE patients with disease satisfying the ACR criteria were classified as non-nephritis or definite nephritis patients based on questionnaire and comprehensive medical record review. Patients classified with definite nephritis fulfilled the criteria of proteinuria (> 0.5 g per 24 hrs), active sediment, or renal biopsy consistent with SLE. 716 (92%) were female. The ethnic distribution was: Caucasian 453 (58.2%), Hispanic 123 (15.8%), Asian 97 (12.4%), African American 75 (9.6%), and other 36 (4.6%). The annual rates of developing nephritis among different gender and ethnic subgroups were derived using Kaplan-Meier estimates.

**Results:** The table shows gender and ethnicity based Kaplan-Meier estimates of the proportion of patients with nephritis at designated time intervals. The curves are significantly different for males vs. females and Caucasians vs. non-Caucasians based on the log-rank test. The curves for Hispanic, Asian and African American subgroups did not differ significantly.

Proportion with nephritis at intervals after SLE diagnosis

	1 year	2 years	3 years	5 years	10 years	P value *
Male	.47	.51	.51	.54	.57	
Female	.20	.23	.24	.25	.30	8.16 x 10 <sup>-7</sup>
Caucasian	.15	.17	.17	.18	.20	
Non-Cauc	.33	.37	.40	.41	.49	6.23 x 10 <sup>-14</sup>

\* P value for log-rank test

**Conclusions:** These results are a significant contribution to the data regarding time to development of nephritis in SLE, and emphasize the important influence gender and ethnicity.

**P111**

**Increased apoptosis level in late stages of rheumatoid arthritis correlates with macrophage number**

**AI Catrina, A-K Ulfgren, L Gröndal\*, S Lindblad, L Klareskog**

*Department of Medicine, Unit of Rheumatology, Karolinska Hospital, Stockholm; \*Department of Orthopaedic Surgery, University Hospital, Uppsala, Sweden*

**Introduction:** Rheumatoid arthritis (RA is a chronic inflammatory disease characterized by synovial hyperplasia and excessive mononuclear infiltration. Altered apoptosis was proposed as a possible mechanism for cell accumulation. *In vitro* experiments showed that monokines are able to inhibit synovial apoptosis in a dose dependent manner. In this study we aim to investigate synovial apoptosis with respect to disease duration, inflammatory cell type and monokines expression.

**Materials and methods:** Synovial biopsy specimens from eleven patients with longstanding RA (mean disease duration 21 years)

and eight with early RA (mean disease duration 5 months) have been investigated. Samples were evaluated for apoptosis (TUNEL method combined with morphologic analysis), cell surface markers (CD3, CD68) and monokine expression (IL1 $\alpha$ , IL1 $\beta$ , TNF $\alpha$  and IL6). Tissue sections were then microscopically analysed using computerised image analysis. Statistical analysis was done using Mann-Whitney test, Spearman correlation test and linear regression.

**Results:** Apoptosis level in RA synovium is significantly higher in late cases compared with early ones ( $P = 0,001$ ), while macrophage population significantly decreases during disease progression ( $P = 0,003$ ). Macrophage score is negatively correlated with apoptosis level ( $R = -0,618$ ;  $P = 0,0088$ ). In contrast, no correlation could be observed between apoptosis and monokine expression or T cell score.

**Discussion:** Low level of apoptosis in early RA cases suggests an ineffective cell death mechanism that ultimately contributes to cell accumulation into the joint and propagation of the inflammatory response. Apoptosis is restored during disease progression, in parallel with a decrease of the macrophage number. These findings suggest apoptosis as a possible marker for early RA and a promising therapy target.

**P112**

**Protein kinase C inhibition dephosphorylates the ribosomal P proteins while inducing apoptosis in Jurkat cells and peripheral human T cells**

**X Wu, S Schatt\* and P Hasler**

*Forschungslabor, Rheumatologische Universitätsklinik, Felix Platter Spital; \*Pränatale Forschung, Universitätsfrauenklinik, Kantonsspital, Basel, Switzerland*

The control of phosphorylation of CK2 target sites has long been a matter of controversy. We have demonstrated that phosphorylation of the ribosomal P protein CK2 sites is reliably measurable by 2D western blotting of whole cell lysates. Physiologically, phosphorylation of the CK2 sites of the P proteins is necessary for the elongation phase of protein translation, which can be used as an indirect parameter of P protein function. Based on previous data showing that crosslinking of CD95 and hyperthermia lead to the dephosphorylation of the ribosomal P protein CK2 phosphorylation sites and decreased protein synthesis, which was associated with the induction of apoptosis in Jurkat cells, we examined whether similar mechanisms are initiated when apoptosis is induced by inhibition of the PKC pathway. In Jurkat cells and freshly isolated peripheral blood T cells, rapid dephosphorylation of the ribosomal P proteins P0, P1 and P2 was induced by low concentrations of chelerythrine, a specific inhibitor of PKC. Neither of the specific PKC activators thymeleatoxin or PMA were able to prevent the dephosphorylation. Inhibition of intracellular Ca<sup>2+</sup> release by TMB-8 also induced dephosphorylation of the P proteins, which is compatible with the requirement of intracellular Ca<sup>2+</sup> for classical PKC isozyme activity. Chelerythrine also induced apoptosis in Jurkat cells, which was prevented by zVAD-fmk and ZnCl<sub>2</sub>, though these agents did not inhibit the dephosphorylation of the P proteins. The effects of chelerythrine were not due to altered CK2 activity, and there was no evidence that the cAMP-dependent PKA, ornithine decarboxylase, or protein phosphatase 2A pathways were involved in signaling leading to P protein dephosphorylation. The dephosphorylation of the P proteins was accompanied by markedly reduced whole cell protein synthesis, which, in parallel with dephosphorylation of the P proteins, was not affected by zVAD-fmk or ZnCl<sub>2</sub>. Freshly isolated peripheral blood T cells showed the same pattern of responses as Jurkat cells, with the exception that chelerythrine did not induce apoptosis in resting T cells.



onset of autoimmunity, and it is a general observation that autoimmune diseases are associated with distinct profiles of autoantibodies. During the last five years there has been a growing interest in finding possible connections between apoptosis and autoimmunity. It has been hypothesized that the recognition, uptake, processing, or presentation of modified self-antigens may promote autoantibody production. At present, many autoantigens have been found that are modified (i.e. cleaved, phosphorylated or dephosphorylated) during apoptosis. Using autoimmune patient sera in immunoprecipitation and Western blotting assays with cell extracts derived from non-apoptotic and apoptotic cells, we identified sera reactivities specific for (novel) modifications of autoantigens.

In this paper we give an overview of our studies on human recombinant autoantibodies derived from patients suffering from rheumatic diseases. Furthermore, we describe the generation of recombinant chicken antibodies specific for human autoantigens that will also be used to study these antigens and (apoptotic) modifications thereof in more detail.

We selected human recombinant antibodies from patient phage display scFv combinatorial antibody libraries (complexity of 107 or higher) derived from peripheral blood or bone marrow lymphocytes of patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and scleroderma (SSc). Next to the human patient libraries we also used chicken libraries made from spleens of animals immunized with 7 human (auto)antigens simultaneously. Screening of both patient and animal derived libraries with recombinant or purified autoantigens resulted in several recombinant monoclonal human and chicken (auto)-antibodies. From our SLE patient libraries, autoantibodies against U1snRNP components U1-A, U1-C, U1-70k and U1-RNA were selected. Moreover, from our SLE libraries we selected anti Ro52 and anti ribosomal P protein antibodies, and from both our SLE and scleroderma libraries we selected anti-La and anti-a-fodrine recombinant antibodies. From our RA patient libraries antibodies specific for RA related peptides were obtained. All human scFv clones obtained were characterized by ELISA, immunoprecipitation assays, western blotting, epitope mapping and of some clones the affinities were measured and competition experiments with patient sera were performed. Sequence analysis was performed to study the germline usage. All chicken clones were sequenced and analyzed by LIA, Western blot and ELISA.

We will discuss characteristics of some of the selected scFv's i.e. epitope mapping, germline gene usage, and competition experiments with patient sera.

The phage autoantibodies selected from autoimmune patient libraries were also analyzed for their specificity for (apoptotically) modified forms of their target autoantigens by Western blotting and immunoprecipitation assays using apoptotic cell-extracts. Some anti-La, anti-70K and anti-RA peptide scFv's recognized (apoptotically)-modified forms of their target antigens.

**Conclusions:** The use of antibody phage display proves to be an extremely helpful technique in studying autoantibodies and autoantigens. Modifications of autoantigens (by apoptosis and/or necrosis) seem to play a major role in the ontogeny of autoimmune diseases. Currently, by using antibody phage display libraries in combination with patient sera we continue our search for possible modifications of autoantigens involved in the ontogeny of autoimmune disease.

## P117

### Diagnostic value of synovial fluid analysis in pigmented villonodular synovitis (PVS)- a proposal of diagnostic criteria.

I Zimmermann-Górska, M Puszczewicz and G Białkowska-Puszczewicz

Department of Rheumatology and Rehabilitation, Karol Marcinkowski, University of Medical Sciences, Poznań, Poland

PVS is an idiopathic lesion that affects the synovial joints, tendons, sheaths and bursa through the production of tumour-like growths. Diagnosis of PVS is difficult. Arthroscopy and biopsy together with microscopic examination are usually a base. According to our experience, cytologic features of synovial fluid are a very useful diagnostic tool in PVS.

**Material and methods:** Synovial fluids (SF) from the joints of 14 patients with biopsy-proven PVS were examined. Moreover in all the patients features of the disease were confirmed in surgical specimens. Synovial fluids were divided into three samples, for physico-chemical analysis, bacteriological and cytologic findings, and placed in sterile tubes. For cytological examination MGG staining was used.

**Results:** SF analysis had revealed an inflammatory character of effusion. In all the cases synovial fluids were bloody and fragments of synovial villi in their sediments were observed as well as multinucleated giant cells, pseudomalignant cell, macrophages with phagocytized hemosiderin, foam cell and a few synoviocytes. Moreover the fat crystals were seen, under polarized light.

**Conclusion:** Cytological features of synovial fluid in PVS are in parallel with results of microscopic examination of joints tissues. In our opinion SF analysis should be the first step in the diagnostic procedure in PVS. We propose the following criteria:

*Major:*

1. the presence of bloody fluid with fragments of synovial villi in sediment
2. macrophages containing hemosiderin
3. pseudomalignant cells

*Minor:*

1. multinucleated giant cells
2. foam cells
3. fat crystals

The diagnosis of PVS can be established if all the major and at least one of the minor criteria are fulfilled.

## P118

### Analysis of anti-Ro52 antibodies in sera of healthy subjects

C Zimmermann, G Fabini, E Höfler, JS Smolen and G Steiner

2nd Department of Internal Medicine, Lainz Hospital and 3rd Department of Internal Medicine, University of Vienna, Vienna, Austria

Anti-Ro/SSA antibodies (ab) are directed to two proteins, Ro60 and Ro52. While anti-Ro60 ab are predominantly found in patients with SLE or primary Sjögren's syndrome, anti-Ro52 ab can be detected also in sera of healthy subjects. These antibodies escape detection by ELISA or immunoblotting and can be found only in immunoprecipitation assays. Recently, an unexpected interaction of Ro52 with IgG has been reported which appeared to occur independently of the antigen binding site. To investigate this unusual interaction, serum IgG was covalently bound to protein A sepharose (PAS) or an anti-IgG agarose column was used and incubated with HeLa cytoplasmic extracts and various Ig fractions as competitors (IgG1-4, IgM, Fab, F(ab)2, Fc, pFc', Fc', C1q). Proteins bound to



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## L6

### DNA therapeutics: a feasible option for treatment of inflammatory diseases?

E Wagner

*Boehringer Ingelheim Austria, Dr Boehringer Gasse 5-11, A-1121 Vienna, Austria*

Recombinant proteins can be very potent, but their therapeutic application can be strongly hampered by inappropriate distribution, dosage, kinetics or toxic effects. Targeted delivery of proteins such as cytokines would be strongly desirable.

DNA therapeutics comprise the delivery of genetic information on a piece of DNA as therapeutic prodrug. This prodrug can be transcribed and translated into a protein (the actual drug) within the target cells, preferably in a tissue-specific and bio-regulated fashion. Basically, two types of nonviral gene transfer systems [1] have been developed: particle-based systems, with DNA packaged into cationic lipids or polymers; and physical techniques which are based on combining DNA with a physical device. Intramuscular administration of naked DNA has already proven as interesting concept for vaccination [2], despite the low efficiency of the method. Two physical device technologies, electroporation and the gene gun, were found to enhance gene expression levels up to 1000-fold over injection of naked DNA alone. This enhancement has also recently been shown by several groups to trigger immune responses against defined antigens in several species [3].

We have generated particle-based systems that can target gene delivery and expression into distant target tissues. We use DNA polyplexes conjugated with cell-binding ligands such as transferrin for receptor-mediated endocytosis. The surface charge of complexes is masked by covalent coating with polyethyleneglycol (PEG). Tumor targeting has been demonstrated in mouse models after systemic administration. With systemically applied tumor necrosis factor (TNF) alpha gene, tumor necrosis and regression of tumors was observed, but no systemic TNF-related side effects. Opportunities to apply local or systemic DNA therapeutics for inflammatory diseases will be discussed.

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## L7

### Chromosome segregation one hundred years after Mendel's rediscovery

K Nasmyth

*IMP, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria*

In eukaryotic cells, replicated DNA strands remain physically connected until their segregation to opposite poles of the cell during anaphase. This "sister chromatid cohesion" is essential for the alignment of chromosomes on the mitotic spindle during metaphase. Cohesion depends on a multisubunit protein complex called cohesin, which possibly forms the physical bridges that connect sisters. Proteolytic cleavage of cohesin's Scc1 subunit at the metaphase to anaphase transition is essential for sister chromatid separation and depends on a conserved protein called separin. We show here that separin is a cysteine protease related to caspases and that it alone can cleave Scc1 in vitro. By replacing one of Scc1's cleavage sites by that for a different site specific protease, we show that cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the separation of sister chromatids and their segregation to opposite cell poles.

## L8

### Genetic analysis of the pentraxin genes in SLE

AI Russell, CA Robertson, S Chadha, DS Cunningham Graham and TJ Vyse

*Imperial College, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*

The aetiology of systemic lupus erythematosus (SLE) is unknown. However, there is good evidence to support a genetic contribution in lupus, including a number of mouse strains that are genetically predisposed to develop lupus. Several groups have published genome-wide mapping studies on multi-case families. More than 15 intervals have been linked with SLE – they are large enough to contain several hundred genes; the aetiological polymorphisms contained within them remain to be established.

We are establishing a large collection of single case nuclear families with the aim of fine mapping the aetiological polymorphisms. Using a candidate gene approach, we have examined several genes, which lie within the linked intervals. First, we identified genetic markers in the candidate genes. The inheritance of the markers in our nuclear families was then tested using the program TRANSMIT which compares the observed and expected rates of transmission of marker alleles (or haplotypes) from parents to offspring. A marked distortion away from random segregation indicates association with disease.

We have hypothesised that genetic variation in the pentraxin genes, C-reactive protein (CRP) and serum amyloid component P (SAP) predisposes to SLE. These two genes are tightly linked on chromosome 1q21-23, a region linked to human SLE. Other evidence implicating these includes the defective CRP response in SLE and the presence of antinuclear autoimmunity in *Sap* knockout mice. We identified five novel single base pair polymorphisms (three in *CRP* and two in *SAP*) and tested these for evidence of association. Individuals from 354 families were studied.



In rheumatoid synovial tissue, (pro)filaggrin was confirmed to be absent, however several deiminated proteins were detected. Among them, only two proteins were highly reactive with AFA. They were identified as the alpha and beta chains of fibrin. Deiminated fibrin therefore appears as the major synovial target of AFA and probably correspond to their genuine target.

In RA patients, the proportion of AFA among IgG was recently found to be largely higher in the synovial interstitium than in synovial fluid and serum, moreover AFA were shown to be secreted by plasma cells of the rheumatoid pannus.

These results strongly suggest that the chronic conflict between the locally secreted AFA / antifibrin autoantibodies and the fibrin deposits particularly prominent in the RA synovium, play a central role in the pathophysiology of RA.

### L13

#### Anti-inflammatory activity of statins: potential use in the anti-phospholipid syndrome

PL Meroni

Department of Internal Medicine, IRCCS Istituto Auxologico Italiano, University of Milan, Italy

**Background:** Hydroxymethylglutaryl Coenzyme A reductase (HMGCoA-red) inhibitors are cholesterol lowering drugs which display pleiotropic effects on several cell types including endothelial cells (EC). Patients with antiphospholipid syndrome (APS) are characterized by the persistent presence of antiphospholipid antibodies (aPL) and by a high incidence of recurrent thrombotic events. aPL have been demonstrated to bind and activate cultured human EC thus contributing to a prothrombotic state. We evaluated the ability of HMGCoA inhibitors to affect the EC activation induced *in vitro* by aPL and in particular by antibodies reacting with the PL-binding protein  $\beta$ 2 glycoprotein I ( $\beta$ 2GPI). Both human monoclonal IgM and polyclonal IgG anti- $\beta$ 2GPI antibodies were used. EC activation was evaluated as adhesion molecule (ADM) expression and cytokine production.

**Methods:** ADM expression was evaluated by a cell ELISA. EC were incubated with human recombinant (hr) IL-1 $\beta$  (50 U/ml), hr TNF $\alpha$  (10 ng/ml), LPS (20 ng/ml) or with human anti- $\beta$ 2GPI antibodies (100  $\mu$ g/ml) for 4 hr for E-Selectin expression and for 20 hr for ICAM-1 evaluation. Cytokine production was investigated by using the RiboQuant<sup>TM</sup> *in vitro* transcription assay to measure IL-6 mRNA expression. As control, EC monolayers were incubated with irrelevant monoclonal or polyclonal antibodies or medium alone. The same experiments were carried out with EC monolayers pre-incubated overnight with fluvastatin or simvastatin (1-10  $\mu$ M) in the absence or presence of mevalonate (100  $\mu$ M). E-Selectin specific NF $\kappa$ B expression was also evaluated by the gel-shift assay.

**Results:** Both statins inhibited in a concentration dependent-manner the ADM expression induced by anti- $\beta$ 2GPI antibodies as well as those induced by the other agonists, being fluvastatin more efficient than simvastatin. Fluvastatin also down-regulated the mRNA expression specific for IL-6 and significantly inhibited E-Selectin NF $\kappa$ B DNA-binding. The simultaneous addition of mevalonate to fluvastatin completely prevented the drug inhibitory effect.

**Conclusions:** These data demonstrates for the first time that statins (and particularly fluvastatin) are able to inhibit an endothelial pro-adhesive and pro-inflammatory phenotype induced by different stimuli including anti- $\beta$ 2GPI antibodies or pro-inflammatory cytokines. Altogether these findings suggest a potential usefulness for statins in the prevention of the APS pro-atherothrombotic state.

### L14

No abstract

### L15

No abstract

### L16

#### The place of mitochondria in apoptosis

G Kroemer

CNRS-ULR1599, Institut Gustave Roussy, F-94805 Villejuif, France

Apoptosis research has recently experienced a change from a paradigm in which the nucleus determined the apoptotic process to a paradigm in which caspases and, more recently, mitochondria constitute the center of death control. Mitochondria undergo major changes in membrane integrity before classical signs of cell death become manifest. These changes concern both the inner and the outer mitochondrial membranes, leading to the dissipation of the inner transmembrane potential and/or the release of intermembrane proteins through the outer membrane. An ever increasing number of endogenous, viral, or xenogeneic effectors directly act on mitochondria to trigger permeabilization. At least in some cases, this is achieved by a direct action on the permeability transition pore complex (PTPC), a multi-protein ensemble containing proteins from both mitochondrial membranes which interact with pro- and anti-apoptotic members of the Bcl-2 family. At present, it is elusive whether opening of the PTPC is the only physiological mechanism leading to mitochondrial membrane permeabilization. Proteins released from mitochondria during apoptosis include caspases (mainly caspases 2, 3 and 9), caspase activators (cytochrome c, hsp 10, Smac/DIABLO), as well as a caspase-independent death effector, AIF (apoptosis inducing factor). Apoptosis inducing factor (AIF) is encoded for by one single gene located on the X chromosome. AIF is ubiquitously expressed, both in normal tissues and in a variety of cancer cell lines.

The AIF precursor is synthesized in the cytosol and is imported into mitochondria. The mature AIF protein, a flavoprotein (prosthetic group: FAD) with significant homology to plant ascorbate reductases and bacterial NADH oxidases, is normally confined to the mitochondrial intermembrane space. In a variety of different apoptosis-inducing conditions, AIF translocates through the outer mitochondrial membrane to the cytosol and to the nucleus. Ectopic (extra-mitochondrial) AIF increases the permeability of the outer mitochondrial membrane, thereby triggering the release of the caspase activator cytochrome c. Moreover, AIF induces nuclear chromatin condensation, as well as large scale (~50 kbp) DNA fragmentation. Thus, similar to cytochrome c, AIF is a phylogenetically old, bifunctional protein with an electron acceptor/donor (oxidoreductase) function and a second apoptogenic function. In contrast to cytochrome c, however, AIF acts in a caspase-independent fashion. The molecular mechanisms via which AIF induces apoptosis, as well as the phenotype of AIF knock-out cells will be discussed.

### L17

#### New approaches to inhibiting TNF production in rheumatoid arthritis: is pathological TNF regulated in the same way as protective TNF?

M Feldmann, B Foxwell, R Maini and F Brennan

Kennedy Institute of Rheumatology Division of Imperial College School of Medicine, London, UK

The success of anti-TNF therapy of rheumatoid arthritis with infliximab (Remicade) and etanercept (enbrel) has prompted us to seek other ways of inhibiting TNF production, and to seek to determine the cellular and molecular mechanisms underlying the excess and prolonged TNF synthesis in RA.



cells were found to have a characteristic biological function; holding lymphocytes underneath and supporting the development and proliferation of these cells. This function named "pseudoemperipolesis" was originally found by Dr Wekerle (1980) in thymus cells of rats and mice, and those fibroblastic cells were named as nurse cells. We established the mesenchymal fibroblastic cell lines from synovial tissue and bone marrow cells in RA patients, and found the pseudoemperipolesis in these fibroblastic cells (nurse-like cells; NLC) just like nurse cells.

We isolated monocytes from the peripheral blood of healthy donors, and incubated with NLC from RA patients (RA-NLC). After 4 weeks of culture, TRAP- positive mononuclear cells with larger cytoplasm appeared. Monocytes cultured in medium alone died within 6 weeks. These TRAP- positive mononuclear cells differentiated into the multinucleated giant cells by incubating with some cytokines even in the absence of RA-NLC. These multinucleated giant cells showed the bone-resorbing activity by culturing on dentine slices. Considering that the significantly higher number of TRAP- positive mononuclear cells and the much more nucleated giant cells with higher bone-resorbing activity could be obtained from the iliac bone marrow of patients with more erosive disease group, RA-NLC could be considered to play important roles in highly activated bone destruction (including severe secondary osteoporosis) of RA patients.

## L22

No abstract

## L23

### Molecular events in cartilage formation and remodeling

Dick Heinegård

Department of Cell and Molecular Biology, Lund University, BMC - Plan C12, SE-221 84 Lund, Sweden

Cartilage extracellular matrix contains a major component of highly anionic proteoglycan contributing fixed charges creating and osmotic environment and a swelling pressure important for resisting pressure load. Another key element is a network of fibers with collagen 2 as the major constituent providing tensile properties and an ability to take up load.

In forming the cartilage matrix the cells produce the macromolecules that constitute the building blocks. These are assembled into the structures of the tissue outside of the cells in a number of specific interactions. An example is the fiber network where collagen molecules form fibrils by interactions where a variety of matrix molecules act as catalysts/chaperons or inhibitors.

Examples of molecules interacting with collagen are particularly found among the leucine rich repeat proteins (LRRP). These include decorin, fibromodulin, lumican and biglycan all with known capacity to bind collagens and influence fibrillogenesis in vitro. This binding occurs via the LRR-domain. Furthermore, the molecules have an additional functional domain, that in the case of decorin carries dermatan sulfate chains capable of interacting with other constituents in the matrix including other collagen fibers thereby crossbridging and creating a fibrillar network covering large parts of the tissue.

In the case of decorin, lumican and fibromodulin, mice with inactivated genes show alterations in collagen fibril assembly indicative of roles at different stages of the process. PRELP binds collagen via its repeat domain and heparan sulfate via a characteristic N-terminal extension. This includes binding heparan sulfate at the cell surface. Chondroadherin binds cells via their  $\alpha 2\beta 1$  integrin. The molecule can actually be isolated from cartilage bound to collagen 2 molecules after activation of endogenous proteinases.

COMP represents a different class of molecules with five identical subunits held together in their N-terminal end. The C-terminal end of each chain has a structure allowing tight and specific interactions with triple helical collagen. There are four sites along the collagen molecule each with a KD of 10<sup>-9</sup>. COMP in vitro has a marked effect in catalyzing the correct assembly of collagen fibers, while not binding to the completed fiber. Thus, the molecule act as a chaperon.

Interestingly COMP is upregulated in early phases of osteoarthritis, where a repair attempt of the damaged tissue is likely to be a component. The molecule or fragments thereof released to synovial fluid and blood, actually serves as an indicator of processes in the cartilage leading to its destruction.

In processes in cartilage remodeling, many of the constituents in the matrix are degraded and lost to surrounding body fluids. This degradation is likely to be a response to remodeling following material fatigue, altered load or growth. It may also occur as part of a pathological process. It is likely that it is coupled to attempts at repair laying down new matrix constituents to produce an adequately functioning matrix. In disease it is apparent that the imbalance between breakdown and adequate repair leads to progressive changes in cartilage composition characteristic for the various stages of the disease.

## L24

### The molecular mechanism of osteoclastogenesis: ODF/RANKL-dependent and independent pathways

T Suda<sup>†</sup>, N Takahashi<sup>\*</sup>, N Udagawa<sup>\*</sup> and C Miyaura<sup>‡</sup>

<sup>\*</sup>Showa University School of Dentistry, Tokyo 142; <sup>†</sup>Medical Culture, Tokyo 171; <sup>‡</sup>Tokyo University of Pharmacy and Life Sciences, Tokyo 192, Japan

It is well established that osteoblasts and bone marrow stromal cells express osteoclast differentiation factor (ODF, also called RANKL) in response to several bone-resorbing factors to support osteoclast differentiation from their precursors. Osteoclast precursors which express RANK, a TNF receptor family member, recognize ODF/RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of M-CSF. Osteoclastogenesis inhibitory factor (OCIF, also called OPG) acts as a decoy receptor for ODF/RANKL. ODF/RANKL is responsible for inducing not only differentiation, but also survival and activation of osteoclasts.

IL-1 and TNF $\alpha$  also play a major role in the pathogenesis of bone resorption induced by inflammation. IL-1 induced osteoclast differentiation by a classical ODF/RANKL-dependent mechanism, indicating that osteoblasts are essential for IL-1-induced osteoclast formation. In contrast, mouse TNF $\alpha$  strongly stimulated differentiation of M-CSF-dependent bone marrow macrophages (M-BMM $\phi$ ) into osteoclasts without any help of osteoblasts/stromal cells. Osteoclast formation by TNF $\alpha$  was inhibited by antibodies against TNF receptor type 1 and 2 (TNFR1 and TNFR2), but not by OPG/OCIF, indicating that differentiation of M-BMM $\phi$  into osteoclasts by TNF $\alpha$  occurs by a mechanism independent of the ODF/RANKL-RANK interaction. IL-1 failed to induce differentiation of M-BMM $\phi$  into osteoclasts.

More recently, we found that lipopolysaccharides (LPS)-induced bone loss did not occur in knockout mice of EP4, a subtype of PGE<sub>2</sub> receptor. This indicates that EP4 signals are involved in the LPS-induced bone resorption. LPS appeared to induce osteoclast formation by two different pathways: one is an ODF/RANK-independent pathway involving TNF $\alpha$ . LPS induces TNF $\alpha$  production through toll-like receptor 4 (TLR4) in macrophages, which in turn directly acts on osteoclast progenitors through TNFR1 and TNFR2 to induce osteoclast differentiation. In this pathway, osteoblasts did not appear to be

