

Evidence for monoclonal expansion of synovial T cells bearing V α 2.1/V β 5.5 gene segments and recognizing a synthetic peptide that shares homology with a number of putative autoantigens

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SUMMARY

A peptide of 15 amino acids derived from the cereal glycine-rich cell wall protein (GRP), sharing a significant homology with Epstein–Barr virus nuclear antigen-1 (EBNA-1), fibrillar and procollagen, stimulated synovial fluid (SF) T cells from juvenile (JRA) and adult (RA) rheumatoid arthritis patients. An overexpression of the V α 2 gene family was found in the SF from patients who responded significantly to the peptide. To investigate in more detail the SF T-cell responses to the GRP peptide, we established peptide-specific T-cell lines and clones from a DR8⁺ positive JRA patient with pauciarticular form. The T-cell clones were phenotyped as T-cell receptor (TCR) $\alpha\beta$ ⁺/CD4⁺ and their clonality was investigated by polymerase chain reaction (PCR) and flow cytometric analysis. TCR sequences from different clones demonstrated that the clones were identical and used the V α 2.1/J α 6 combined with V β 5.5/J β 2.7 gene segments. Interestingly, direct sequencing of the V α 2 family PCR product obtained from cDNA prepared from freshly isolated SF mononuclear cells identified the same TCR sequence as that used by the clones, suggesting the monoclonality of SF CD4⁺ T cells bearing V α 2.1/J α 6 gene products. The present data suggest a recruitment and expansion of a SF T-cell subpopulation, and also support the hypothesis that autoimmune diseases can be triggered by protein epitopes with crucial amino acids homologous to self-proteins.

INTRODUCTION

Humoral and cellular immune responses to micro-organism proteins or self-proteins (e.g. collagen, myelin basic protein) have been implicated in the pathogenesis of autoimmune diseases.^{1–6} In the last few years it has become clear that a significant number of T cells in healthy humans can be triggered by certain antigens suspected to be involved in the induction of autoimmune diseases.^{2,6} The strong association between rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA) with certain major histocompatibility complex class II specificities^{7–9} supports the concept of a role for T cells in these diseases. In the past, several studies using restriction fragment length polymorphism (RFLP) or semi-quantitative polymerase chain reaction (PCR) techniques and subsequent sequencing have yielded some evidence of oligoclonal expansion of T cells

in the synovium compartments of RA and JRA patients.^{10–15} However, this type of analysis does not correlate T-cell receptor (TCR) gene segment usage with antigen specificities. Thus, the nature of the disease-related peptide(s) still remains to be determined. In order to answer this question, many groups have analysed the synovial fluid (SF) and peripheral blood (PB) T-cell response to a limited number of suspected antigens.^{4,5,16,17} This type of analysis requires prior structural information about the antigen being used. This constitutes a major limitation in the study of autoimmune diseases, in which T cells are believed to play a major role in the pathogenesis. However, tracks of the aetiological agents responsible for the activation of autoreactive T cells are 'imprinted' in patient serum as a specific antibody response.¹⁸ Therefore, this antibody response can provide important leads for the identification of T-cell epitopes.

The discovery of random peptide phage libraries¹⁹ has made the dissection of this humoral response possible.^{20,21} This novel strategy does not require prior structural information about the antigen(s). In addition, it can identify both linear and conformational epitopes on proteins of interest.^{22,23} To dissect this humoral finger 'imprint' in rheumatoid arthritis patients, we have previously screened a random nanopeptide phage library with a pool of sera obtained from RA patients. Series of nanopeptides reacting with immunoglobulins from RA patients have been

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Abbreviations: CDR, complementary determining region; EBNA-1, Epstein–Barr virus nuclear antigen-1; GRP, glycine-rich cell wall protein; SF, synovial fluid.

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identified.²⁰ Antibodies to the selected peptides were also increased in JRA patients with poly- and pauciarticular forms.

One of the selected peptides (pep1: ADGGAQGTA) showed homology with the Epstein-Barr virus nuclear antigen-1 (EBNA-1) protein and the cereal glycine-rich cell wall proteins (GRPs). The examination of the amino acid sequence of GRP 1.8²⁴ showed the presence of many glycine/alanine repeated sequences similar to EBNA-1 and cytokeratins.

In the past, many studies have been performed with the EBNA-1 protein. Antibodies reactive with synthetic peptides from the EBNA-1 protein were found to be elevated in certain autoimmune diseases.²⁵ However, GRP 1.8 is a ubiquitous food protein that has received no attention, despite its high homology with cytokeratins and other self-proteins. In order to investigate a possible T-cell response, a 15-amino acid synthetic peptide (GGYGDGGAHGGGYGG) was synthesized and its ability to stimulate T cells from JRA and RA patients was investigated. In this report we present molecular evidence for a monoclonal expansion of synovial T cells bearing V α 2/V β 5.5 gene segments specific for this peptide. Our results demonstrate for the first time that peptides selected from random libraries by using autoimmune sera could be useful leads for the identification of T-cell epitopes.

MATERIALS AND METHODS

Patients

Paired SF and PB samples were collected from patients with a diagnosis of JRA and RA who fulfilled the American Rheumatism Association diagnostic criteria.²⁶ The JRA patient with the pauciarticular form, which was investigated in detail, had an active seronegative disease. She was operated on for synovectomy of both knees. Histology showed a hypertrophy of the synovial membrane with chronic inflammation typical of rheumatoid arthritis.

Synthetic peptides

A synthetic peptide of 15 amino acids (GGYGDGGAHGGGYGG) derived from the GRP 1.8 (residue 438–452),²⁴ and an irrelevant synthetic peptide (SAAPGQKVTISCSG), were synthesized and high-performance liquid chromatography (HPLC) purified (Public Health Research Institute, New York, NY).

Generation of T-cell clones

Peptide-specific T-cell clones were generated by stimulating SF mononuclear (MN) cells from a DR8⁺ JRA patient with the pauciarticular form. Cells were stimulated with 20 μ g/ml of the GRP peptide for 10 days in RPMI supplemented with antibiotics and 10% fetal calf serum (FCS). The cells were then stimulated further with the GRP peptide and interleukin-2 (IL-2) for 14 days. After 24 days, cloning was performed by limiting dilution (1 cell/well) in the presence of irradiated autologous PB MN cells (10⁵ cells/well), peptide and IL-2 for 3 weeks. Growing clones were restimulated and expanded further with the GRP peptide, IL-2 and autologous irradiated PB MN cells.

Proliferation assays

The proliferation response of SF MN and PB MN cells to the GRP peptide and control antigens was investigated as described previously.⁵ The GRP and control peptide were

used at a final concentration of 20 μ g/ml and assayed on 10⁵ cells/well. After 5 days of stimulation the cells were pulsed with [³H]thymidine and harvested 18 hr later. All results were expressed as mean counts per minute (c.p.m.) in triplicate. The stimulation index (SI) was defined as (test c.p.m. – control c.p.m.)/control c.p.m., where test c.p.m. = [³H]thymidine incorporation by cells stimulated with GRP peptide, and control c.p.m. = [³H]thymidine incorporation by unstimulated cells. The proliferation of the two T-cell clones (3B2 and 10H1) was analysed by [³H]thymidine incorporation after stimulation with GRP peptide. In these experiments twenty-five thousand cells (3B2 or 10H1) were incubated with 50 000 autologous feeder cells plus peptide for 48 hr, pulsed with [³H]thymidine, and harvested after another 18 hr.

Analysis of TCR V gene repertoire by PCR

The TCR $\alpha\beta$ usage in the synovium was investigated as previously described by semi-quantitative PCR.¹² Briefly, each V α and V β family was amplified using V α - or V β -specific primers^{12,13,27} combined with C α or C β primers. As a positive control for amplification, C α and C β were co-amplified with each V α and V β gene family, respectively. The PCR products were transferred to a nitrocellulose membrane and hybridized with an internal ³²P C α or C β primer. PCR products of the appropriate size were excised from the gel and quantified by liquid scintillation counting. The PCR values were calculated by dividing the radioactivity (c.p.m.) contained within each V β or V α PCR product by their appropriate internal controls.¹² The sequences of V α and V β primers have been described elsewhere.^{12,13}

Preparation of RNA, cDNA synthesis

Total RNA was isolated from SF MN or PB MN cells according to Chomczynski & Sacchi,²⁸ and reverse transcribed using the first strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). Preparation of cDNA from PB MN cells stimulated with the GRP peptide for 20 days was performed with a method developed for sensitive preparation of cDNA for PCR amplification, in which reverse transcription is performed on cells boiled in diethyl pyrocarbonate (DEPC) to inactivate RNases.²⁹ This protocol can rescue mRNA from a low number of activated T cells.

Sequencing and cloning

Following amplification using V α - or V β -family specific primer, the PCR products were analysed by 1.5% low-melting agarose gel electrophoresis, cut from gels and then isolated by the gene clean method (BIO 101 Inc., La Jolla, CA). Following purification, PCR products were sequenced directly with the dideoxy chain termination method using V α - or V β -family specific primer and T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH). The same PCR products were sequenced using C α or C β primers. In the case of cloning, PCR products were blunt ended, kinased and then ligated into *Sma*I-cleaved pGM10. Following cloning and screening, single-stranded DNA was isolated from each positive recombinant clone, and then sequenced by the dideoxy chain termination method using M13 universal primer and the Sequenase Version 2.0 kit (United States Biochemical Corp.).

Phenotypic analysis of T cells

Flow cytometric analysis of the cloned T cells was performed

using standard procedures, as described previously.³⁰ Briefly, 10^5 cells were resuspended in 50 μ l cold Hanks' balanced saline solution (HBSS) containing 0.02% sodium azide in V-bottomed plates, and the following monoclonal antibodies (mAb) were added in saturating concentration for 30 min in ice: anti-TCR $\alpha\beta$ -fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE) (MT310), anti-CD8-FITC (DK25) (all from Dakopatts, Glostrup, Denmark) and unconjugated anti-V α 2 (Ig G-2a) (T Cell Sciences, Cambridge, MA). After washing three times, V α 2⁺ cells were stained with PE-conjugated secondary goat anti-mouse IgG2a antibodies (Southern Biotechnology, Birmingham, AL) for another 30 min, washed and analysed by a Becton Dickinson FACScan flow cytometer and LYSYS II software (Becton Dickinson Immunocytometry Systems, San José, CA).

RESULTS

T-cell response to the GRP peptide

The T-cell response of paired PB MN and SF MN cells from RA and JRA patients and a healthy control group to GRP peptide was investigated. SF MN cells from three out of 11 RA patients and four out of 20 JRA patients showed a significant proliferative response (SI \geq 3) to the GRP peptide compared to the control peptide, as well as to tetanus toxoid. None of the 15 healthy adults and children responded.

Figure 1 shows an example of the proliferative responses of SF MN and PB MN cells from the JRA and the RA patient who responded the most. As can be seen, a SF T-cell response to the GRP peptide was evident, while only a very weak response was noted in the PB of the JRA patient.

TCR usage by freshly isolated T cells from SF and PB of the JRA patient

In order to investigate in more detail the TCR diversity in SF from the two patients presented in Fig. 1, we first examined the SF TCR usage by semi-quantitative PCR using V gene family-specific primers, as described previously.¹² Figure 2 shows a very marked restriction in the V gene usage by the SF T cells from the JRA patient, with overexpression of V α 2 and V β 5 gene families. This restricted profile was reproducible. V α 2 was also over-represented

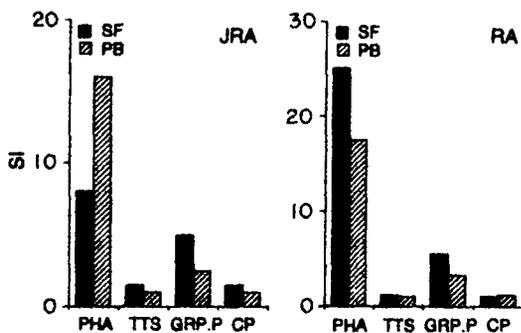


Figure 1. Proliferative responses of PB MN and SF MN cells from a JRA and a RA patient to different stimuli. (PHA, phytohaemagglutinin; TTS, tetanus toxoid; GRP.P, GRP peptide; CP, control peptide). Results are expressed as SI, and are the mean of 2 independent experiments.

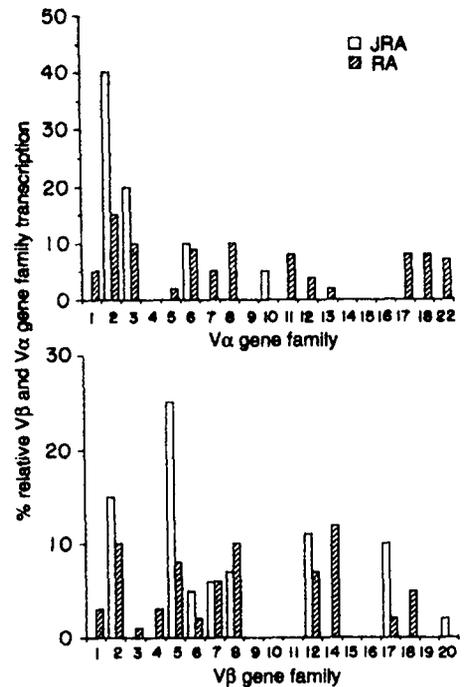


Figure 2. Normalized PCR values of TCR $\alpha\beta$ gene family in freshly isolated SF MN cells from a JRA and a RA patient. The PCR amplification (35 cycles) was performed using each of the indicated V α or V β family-specific primers. The PCR values were calculated as described previously.¹⁶

in the SF from the RA patient. All the V α gene families were detected in the PB from both patients (data not shown). Furthermore, PCR values of V α 2 gene expression by the PB MN cells from both patients were found to be less than 6%.

Analysis of the peptide-specific T-cell line

To determine the molecular and cellular characteristics of T cells that responded to the GRP peptide, we established, from the SF of the JRA patient described in Figs 1 and 2, a T-cell line by stimulation with the GRP peptide. After 20 days of peptide stimulation we investigated the V α TCR usage in the line using V α 2, V α 3, V α 6 and V α 10 family-specific primers combined with a Ca primer. After 35 cycles of amplification, only V α 2 PCR products could be detected. We did not have the chance to analyse the freshly isolated SF T cells, but double staining of a short-term line (only 10 days of stimulation) with TCR $\alpha\beta$ ⁺ V α 2⁺ CD4⁺-specific antibodies demonstrated that approximately 76% of the cells were CD4⁺ V α 2⁺ T cells (Fig. 3a), while 2.5% of PB MN cells were stained with V α 2 antibodies (Fig. 3b).

Generation of peptide-specific T-cell clones

After further stimulation of the line with the peptide and IL-2 (20 days), the T-cell line was cloned by limiting dilution, as described in the Materials and Methods. Six clones were obtained. Two of them were selected and characterized further. Both clones responded to the GRP peptide (SI \geq 15) compared to the control peptide (Fig. 4). These clones

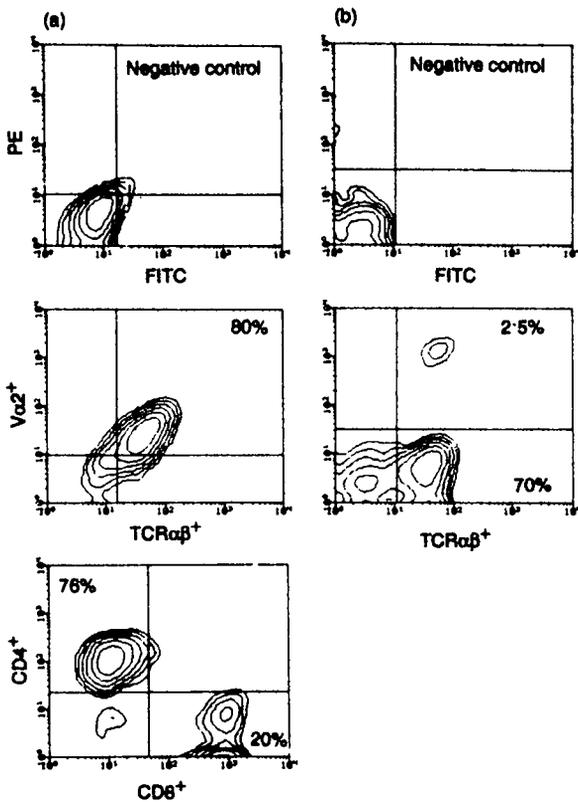


Figure 3. Flow cytometric analysis. (a) Analysis of a short-term SF T-cell line specific for the GRP peptide. The SF MN cells from the JRA patient described in Fig. 2 were cultured in the presence of GRP peptide for 10 days. The cells were phenotyped by two-colour immunofluorescence with anti-TCRαβ-specific mAb and anti-Vα2-specific mAb, or with anti-CD8-specific mAb and anti-CD4-specific mAb. (b) Analysis of freshly freshly isolated MN cells from the PB of the same patient. The cells were stained and analysed as in (a).

were phenotyped as TCRαβ⁺, CD4⁺ and Vα2⁺ T cells (Fig. 5a, b).

Analysis of the TCR gene usage in the peptide-specific clones

To determine the TCR Vβ usage in these two peptide-specific clones, cDNA was prepared and PCR amplification performed using Vβ family-specific primers. Both clones used the Vβ5 gene segment.

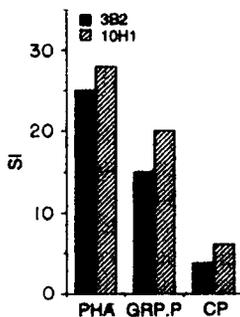


Figure 4. Proliferative responses of clones 3B2 and 10H1 to PHA, GRP peptide (GRP.P) and control peptide (CP). The results are expressed as SI and are the mean of two independent experiments.

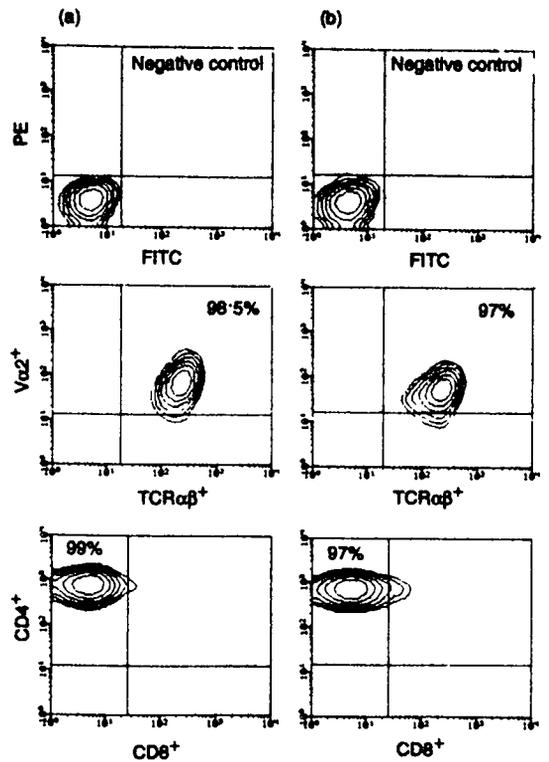


Figure 5. Flow cytometry analysis of GRP peptide-specific clones. The T-cells clones 3B2 (a) and 10H1 (b) were stained with anti-TCRαβ-specific mAb and anti-Vα2-specific mAb, or with anti-CD8-specific mAb and anti-CD4-specific mAb, and analysed by flow cytometry as in

As the CDR3 region encoded by the V-D-J and V-J junction of TCR is thought to be involved in antigen recognition, the Vα and Vβ TCR junctions from the two clones were determined by sequencing. Our data demonstrated that both clones were identical and used the Vα2.1/Jα6 gene segments combined with Vβ5.5/Dβ1.1/Jβ2.7 gene segments (Fig. 6). Although the specificity of the four other clones was not investigated in functional assays, their TCR gene segments had the same sequence as the peptide-specific clones. It is important to note that the JRA patient from whom the T-cell line and clones were derived had a humoral response against pep1, but not the GRP peptide (data not shown).

Analysis of D and J segment usage by the peptide-specific line and freshly isolated SF T cells

The V-J and V-D-J junctional sequences are unique to each T-cell clonotype and contribute to TCR diversity. In order to understand the type of T-cell response to an endogenous antigen that resembles the GRP peptide, we first determined the TCR usage by the long-term peptide-specific line (20 days of stimulation). Following PCR amplification, using a Vα2- or a Vβ5-family specific primer, the PCR products were purified and then sequenced directly. The sequence data indicated that all T cells in the line used the same sequences as the clones, demonstrating that the peptide-specific T-cell line was homogeneous.

To investigate further whether only one single T cell with

polyclonality of SF T cells bearing V α 6 gene products. Thus the combination of PCR amplification using each TCR family-specific primer and direct sequencing was an approach that could dissect the clonality of T cells at the site of inflammation.

The present results have several important implications for current studies of rheumatic diseases and the molecular mechanism of peptide and major histocompatibility complex (MHC) recognition. Perhaps the most important is the detection of a specific clonal expansion of the synovial T cells bearing V α 2.1/V β 5.5 gene products, indicating the involvement of MHC complex-restricted autoantigen recognition, rather than a 'superantigen' in the pathogenesis of JRA.¹⁴ This was supported by the fact that the T-cell response to GRP peptide could be blocked by the addition of anti-DR antibody (data not shown). Our data imply that an endogenous antigen that resembles the GRP peptide may be responsible for the stimulation of SF T cells bearing V α 2.1/V β 5.5 gene products in this particular JRA patient. Based on an homology search (Table 1) one could suggest that this particular T-cell clone may react with a peptide derived from fibrillar collagen, heat-shock proteins (hsp) or unidentified SF or chondrocyte proteins. The proliferation data presented in Fig. 1 indicate that T cells specific for the GRP peptide were not expanded in PB of the JRA patient, since the T-cell response was negative. This observation suggests a selective recruitment of newly stimulated peripheral blood T cells to the synovium. The *in vivo* expansion of T cells bearing V α 2.1/V β 5.5 gene products was supported by semi-quantitative PCR analysis of freshly isolated SF T cells (Fig. 2), and analysis of PB MN cells by flow cytometry following staining with anti-V α 2-specific antibodies. As mentioned in the results section, we did not have the chance to analyse by flow cytometry the freshly isolated SF T cells

from the JRA patient, but analysis of a short-term peptide-specific SF T-cell line indicated that 80% of T cells stained with anti-V α 2-specific antibodies. The prevalence of V α 2 T-cell expansion in the synovium, but not in the peripheral blood, has been observed previously in JRA and RA.^{12,31}

Examination of the amino acid sequence of the GRP 1.8 by Swiss Protein program showed the presence of many glycine/alanine repeated sequences²⁴ similar to EBNA-1 and human cytokeratins. This observation may indicate a possible molecular mimicry. The possibility that autoimmune diseases could be induced through antigen mimicry of T-cell epitopes was initially documented in a study on hsp. Members of hsp families are remarkably conserved.³² There is more than 50% sequence identity between bacterial and mammalian counterparts. Moreover, hsp molecules contain significant stretches of complete sequence identity. A T-cell clone specific for a peptide representing the amino acid sequence 241–255 of the mycobacteria 65 000 MW protein, responded to the equivalent human sequence.¹⁶ The proliferative response of this T-cell clone was comparable to the GRP-specific T-cell clones. On the basis of many studies, the molecular mimicry for the induction of autoimmune diseases requires only a few randomly positioned amino acid residues of self-peptides.^{33–36} For example, a polyalanine peptide with only five native myelin basic protein residues induced autoimmune encephalomyelitis (EAE).³⁵ Thus, a peptide different from the immunizing peptide can fulfil the requirements, as long as the peptide contains the critical MHC and TCR contact residues. As only four or five native residues in a peptide were able to induce EAE, it is conceivable that PB T cells bearing the V α 2.1/J α 6 and V β 5.5D β 1.1/J β 2.7 could be stimulated with a peptide derived from EBNA-1 protein, haemagglutinin of influenza virus

Table 1. Homology of GRP peptide with antigens

GRP peptide	GGYGDGGAHGGGYGG
GRP 1.8 (436–454)	SGGGYGDGGAHGGGYGGGA
EBNA-1 nuclear protein (425–443)*	GGYGDGGAHGGGYGG : : : : : GRRGYGDGRRRKKGGWGKH
hnRNP A2 (266–284)*	GGYGDGGAHGGGYGG : : : : GRGGYGGGGPGYGNQGGGY
Hemagglutinin of influenza virus (a and b) (238–256)*	GGYGDGGAHGGGYGG : : : : : : VRYKKRGAAGGGWYGMV
Fibrillar collagen (247–265)*	GGYGDGGAHGGGYGG : : : RRGGYGRKGRAGAVGDVGD
Procollagen α 1 (V) chain precursor (327–345)*	GGYGDGGAHGGGYGG : : : : : : ANYDTYGGRRRKGKYGRA

: Conserved substitution.

* Residue numbers according to the Swiss Protein Database.

(Table 1). However, this particular SF T-cell clone could be derived from the gut following stimulation with GRPs. Immunological hyper-responsiveness of the gut mucosa to luminal antigens may subsequently produce sufficient stimuli to perpetuate joint diseases. Our data may therefore clarify much of the proposed link between gut involvement in inflammatory joint diseases.^{37,38} The lymphocytes in the chronically inflamed synovium may originate in the gut. In all the above proposed scenarios T cells were recruited to the joint and proliferated there in response to homologous peptides derived from the synovium proteins. The role of the GRP-responding SF T cells in the pathogenesis of RA or JRA is not known. However, these dominant SF T cells may contribute with other SF T cells to the synovitis seen in the JRA patient investigated in this study. The T-cell response to GRP peptide was found in only a few patients, as may be expected for a single epitope. However, we think that more patients may respond to the GRPs. Thus, further detailed studies of T-cell and B-cell responses to GRPs, peptides as well as potential autoantigens, may help towards the identification of SF T-cell specificities. In addition, the use of a variable region family-specific primer in the PCR technique, followed by direct sequencing of the amplified PCR products, as demonstrated in this study, is a strategy that may allow the analysis of T-cell clonality at the sites of inflammation.

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