Rheumatoid arthritis: autoreactive T cells recognizing a novel 68k autoantigen

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Abstract

Objective—A 68k autoantigen has been identified by specific antibodies from patients with rheumatoid arthritis (RA). This study considered whether or not this antigen is a target for T cells and thus may play a part in T cell mediated immunopathology of active RA.

Methods—The 68k antigen was isolated and used in a nitrocellulose bound form to stimulate T cells. Proliferation of T lymphocytes of peripheral blood as well as synovial fluid was measured.

Results—Peripheral blood T cells specifically proliferating against the 68k antigen were detected in 19 of 27 patients with RA (70%). For T cells isolated from peripheral blood, proliferation peaked on day 10. When T cells were isolated from actively inflamed synovial fluid, the proliferation kinetics shifted to a peak on day 3. Blockade of HLA class II antigens resulted in an increase of proliferation in the case of HLA-DP. Applying HLA-DP specific antibodies capable of inhibiting antigen presentation mediated by this molecule, T cells of 17 of 27 RA patients (63%) proliferated to a higher extent than with the 68k antigen alone. The phenomenon that an increased proliferation occurred upon blockade of a particular HLA class II family member was also demonstrated for DQ and DR: the 68k antigen likewise stimulated T cells restricted for DP or DQ, respectively.

Conclusions—The novel 68k antigen is a target of both T and B cellular immune responses and as such could play a part in the immune dysfunction of RA. The finding that blocking of certain HLA class II molecules functioning in antigen presentation (for example, via HLA-DQ) results in a higher instead of lower proliferation in vitro, may argue for the presence of antigen specific suppressive T cells.

A large body of evidence indicates rheumatoid arthritis (RA) to be an autoimmune disease that is T cell mediated and maintained in the microenvironment of the joint. The synovium is infiltrated with activated T cells and macrophages. In inactive arthritis, nevertheless, most T cells seem to be non-specifically recruited into the inflamed joint, which is also believed to be the case in RA. Low frequencies of antigen specific T cells ranging from 1:10³ to 1:10⁴ are well documented for a variety of other autoimmune diseases. The frequency of antigen specific T cells rises to 1:10⁰ or 1:10¹ in the diseased tissue. In rat, adjuvant arthritis can be transferred into naive genetically susceptible animals by as few as 10⁵ autoreactive T cells specific for the disease triggering antigen. Considering that the actual numbers of T cells migrating into the joints may be considerably lower, suggests that very few antigen specific T cells can initiate and maintain a chronic arthritis with immunohistological similarities to RA. The low frequencies of antigen specific T cells should make it difficult to detect them by analysis of T cell receptor genes. They might only be detectable using the corresponding antigen.

Studies on transforming growth factor β (TGFβ) mediated bystander suppression might help to circumvent studying T cells directed to the disease specific self-antigen in order to treat autoimmune diseases. Nevertheless, autoreactive disease specific T cells might not be affected by the otherwise suppressive effects of TGFβ. It is therefore of particular relevance to determine their mode of action to analyse the pathomechanism of the disease. Zhang and colleagues, for example, demonstrated the existence of myelin basic protein (MBP) reactive T cells and anti-clonotypic suppressive T cells of which most of the latter were CD8+.

Suppression required direct contact with the MBP specific T cells and was not cytokine mediated. These findings may apply as well for RA and will be a promising target to challenge with an RA specific antigen. T cell responses to the 65k MT antigen seem to be increased in synovial fluid of patients with chronic inflammatory arthritis, but not specifically those from RA patients. Recently a 68k autoantigen could be identified by our group as the target of antibodies occurring specifically in RA patients, but not in patients with other rheumatic diseases or healthy controls. The 68k antigen is probably ubiquitously expressed in humans or even in mammals, as it was detectable in any tissue analysed by immunoblotting (synovium, liver, spleen, HeLa cells, serum). Immunofluorescence studies located the antigen to the cytoplasm or endoplasmic reticulum. The antigen is O-glycosylated and shows an isoelectric point of 5.1. The high disease specificity (99%) of the anti-68k antibody combined with a sensitivity of 66% make the corresponding 68k antigen an interesting target of autoreactive T cells to investigate. It might be a good candidate antigen to identify activated disease related T cells of low...
frequency. In this study the T cell immune response against this antigen is analysed to examine its functional relevance for the pathogenesis of RA. Therefore, the 68k antigen was purified in a form that is biologically active in nanogram quantities so that it could stimulate T cells in a proliferation assay. It was analysed if the antigen could stimulate T cells in an RA specific fashion and if T and B cellular immune response to the 68k antigen were associated in a particular patient.

**Methods**

**PREPARATION OF THE ANTIGEN**

Total protein was prepared from HeLa cells as described. The material was separated by SDS-PAGE and blotted to nitrocellulose (NC). After identifying the 68k band by RA specific antibodies, the rest of the band was cut out and processed according to Abou-Zeid *et al* with minor alterations. Briefly, this entails solubilisation of the material in DMSO and incubation for one hour at room temperature to sterilise it. From this point on all procedures were carried out in sterile solutions and materials. Subsequently the NC bound protein was precipitated with a 50 mM carbonate/bicarbonate buffer to obtain very fine particles, which can easily be taken up by antigen presenting cells. The material was washed twice with cell growth (CG) serum free tissue culture medium (purchased from Vitromex, Vilshofen, Germany) and finally resuspended in 5 ml medium per 160 mm × 1 mm 68k NC band. Fifty µl per well of this suspension were applied to a 96 well microtitre plate. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immunoreactivity with RA serum served as control antigen. A further control was NC without blotted protein. A 64k protein band reactive with the serum of some RA patients and also some healthy donors served as control antigen in kinetic studies.

**PATIENTS’ CHARACTERISTICS**

Twenty seven patients attending the Rheumatology Unit of the University of Düsseldorf were diagnosed as RA according to the ARA criteria. Blood and synovial fluid samples were obtained after consent by the patients. The 27 RA patients are ordered and numbered according to their responsiveness to the 68k antigen. Fifteen received disease modifying drugs (DMARDs) (nos 1-3, 9, 10, 13, 15-22, 25). All but two (nos 7 and 24) were taking low dose corticosteroids (< 10 mg prednisone per day). Fifteen were anti-68k antibody positive (nos 2-5, 7, 9, 11-12, 14-18, 21-22, 25-27). Fifteen had raised C reactive protein concentrations (nos 1-2, 5-8, 13, 15-17, 18-19, 21-23, 25). Eighteen had a raised erythrocyte sedimentation rate (nos 1-2, 4-8, 12-16, 19, 21-23, 25-26). Two were rheumatoid factor negative (nos 12 and 13), 25 were rheumatoid factor positive. Samples from eight healthy blood donors, age and sex matched with RA patients, were used as controls. RA patients: 19 female, 8 male, mean age 45; healthy donors 5 female, 3 male, mean age 42.

**PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)**

Fresh heparinised blood from RA patients or from healthy laboratory personnel was mixed with phosphate buffered saline (1:1) and carefully layered on Ficoll. This was spun at 900 g for 20 minutes at room temperature. Mononuclear cells could be harvested from the interface at the high and low density zones. Mononuclear cells from peripheral blood and synovial fluid were 95% lymphocytes and 5% monocytes. Viability was 98% as determined with trypan blue exclusion. Cells were counted and frozen in 11% DMSO, 50% fetal calf serum in RPMI 1640 at aliquots of $5 \times 10^6$ cells and stored in liquid nitrogen.

**PREPARATION OF SYNOVIAL FLUID MONONUCLEAR CELLS (SFMCs)**

Fresh heparinised synovial fluid from RA patients was either directly processed like peripheral blood or incubated firstly with 1% trypsin and 0.5 µg/ml of monoconal anti-HLA class II antibodies were applied. Anti-HLA monomorphic antibodies were B7.21.2 specific for HLA-DP, Tü22 for HLA-DQ, and Tü36 for HLA-DR. They have been described as blocking antigen presentation restricted by a particular HLA family member. The antibodies do not by themselves stimulate T cell proliferation either in the presence or absence of specific antigen. They do not cross react with the 68k, the control antigen, or with NC free of protein. As controls, monoclonal anti-β tubulin antibodies were applied, which had no effect on proliferation rates. After incubation at 37°C in the presence of 5% CO₂, [³H]-methyl-thymidine was added on day 2, 7, 9 or 14. The radioactivity incorporated into the DNA was determined by scintillation counting as a measure for proliferation after 16 hours of incorporation.

**STATISTICS**

The proliferation data derived from stimulation with the 68k antigen in the absence or presence of anti-HLA class II antibodies were assessed for normal distribution by the $\chi^2$ test. The data did not follow a normal distribution for the 68k antigen ($p < 0.025$) or for the blocking results with anti-DP and anti-DQ ($p < 0.001$), and the anti-DR antibodies ($p < 0.01$).
Stimulation of cells derived from healthy donors against the 68k antigen was the same as against CG medium and set 100%. T cell stimulation was considered as such when the mean incorporation rate of a particular rheumatic probe differed significantly (p < 0.0001, Whitney U test) from healthy controls; this was the case from 120% on. Inhibition of T cells was significant with p < 0.0001 from below 80%. The patients were numbered according to their responsiveness to the 68k antigen. This numeration is kept for the experiments with the monoclonal anti-HLA antibodies.

Proliferation peaks were compared by Fisher's exact test.

Results

DETECTION OF 68K SPECIFIC T HELPER CELLS IN PERIPHERAL BLOOD OF RA PATIENTS

The 68k antigen was prepared in a biologically active, NC bound form as described: it was estimated that nanogram quantities of the antigen applied per well could stimulate their specific T cells from 10⁵ cells seeded.

In a kinetic study, proliferation of RA patient derived mononuclear cells against the 68k antigen was determined by [H]-thymidine incorporation after 2, 4, 7, 9, and 14 days of incubation. Proliferation was found to peak on day 10 with PBMCs of four RA patients (fig 1A). For three of these patients the peak significantly (p < 0.001; Fisher's exact test) shifted to day 3 when SFMCs were applied, while for the other patient it remained to be day 10 (fig 1B), although proliferation at day 3 was also increased compared with day 3 with PBMCs. Five other RA patients of whom no PBMCs were available, showed a peak at day 3 and two on day 10 (data not shown).

Proliferation of RA patient or healthy control derived mononuclear cells against a 64k control antigen peaked at day 5 (fig 1A and B) and thus differed significantly (p < 0.001; Fisher's exact test) from the kinetics against the 68k antigen.

PBMCs were isolated from blood samples of RA patients and healthy controls and stimulated with the 68k or control antigen to analyse whether 68k specific T cells were present in RA patients and whether they were unique to these patients. T cells isolated from the blood of RA patients could be maximally stimulated on day 10 with the 68k antigen, but not with a 70k control antigen or NC without antigen. T cells isolated from blood of healthy donors neither reacted with the 68k nor control antigen or antigen free control. T cells specific for the 68k antigen could be determined in 19 of 27 (70%) of RA patients (fig 2). Although most of the patients positive for 68k specific T cells were also anti-68k antibody positive, about half of the patients negative for 68k specific T cells were also 68k antibody positive; this association was not statistically significant (p > 0.5, Whitney U test). Likewise no correlation to disease activity could be seen.

DETECTION OF HLA CLASS II RESTRICTED SUPPRESSIVE T CELLS SPECIFICALLY STIMULATED BY THE 68K ANTIGEN

Studies demonstrating the existence of DQ restricted T cells of the suppressor type led us to analyse whether such T cells also play a part in autoimmunity to the 68k antigen for three reasons. Firstly, RA patients are divided into three subsets according to the net T cell response against the 68k antigen: one set harbouring T cells that can be stimulated, one that can be inhibited, and another one that
remains unaffected. Secondly, the rather low incorporation rates, as well as the proliferation peaking on day 10 of stimulation, argued for a response distinct from a regular T helper cell response. Finally, the 68k antigen is a major target of RA specific autoantibodies.

Therefore, monomorphic anti-DQ antibodies (Tü22) were added to the proliferation assay to prevent antigen presentation via this particular HLA. Thus, it was considered if DQ restricted 68k specific suppressive T cells do exist. The data were again not normally distributed as determined by the χ² test. In fact, the inhibition of this pool of cells by anti-DQ antibodies resulted in an increased proliferation of the remaining pool of DP and DR restricted cells upon stimulation with the 68k antigen in some RA patients. Ten of 27 RA patients (37%) proliferated to a significantly (p < 0.001) higher degree compared with the response in the absence of anti-DQ antibody (fig 3B). Eleven (41%) proliferated to a significantly lower degree (p < 0.001) and six (22%) showed no significant effect.

In subsequent experiments monomorphic anti-DP (B7.21) and anti-DR (Tü36) antibodies were applied. In the presence of blocking anti-DP antibodies, a significantly (p < 0.001) increased stimulation could be seen in 17 of 27 RA patients (63%) (fig 3A), and in 13 of 27 (48%) with anti-DR antibodies (fig 3C). A significantly (p < 0.001) decreased stimulation was seen in eight (fig 3A) and in nine (fig 3C) patients, respectively. No significant effect was observed in two (fig 3A) and in five patients (fig 3C).

It became evident that a single person harboured DQ as well as DP restricted 68k specific T cells of the suppressive type (table 1). In fact only five subjects showed 68k specific suppression restricted to only one HLA family. Another seven showed no preferential suppressor subset. The remaining 15 RA patients displayed proliferative T cells restricted by two or three HLA class II antigens (table 1).

**Table 1** RA patients harbouring 68k specific T cells. RA patients (n=27): HLA restrictions of suppressive T cell subsets

<table>
<thead>
<tr>
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**Figure 2** T cell proliferation upon stimulation with the 68k antigen. In 19 of 27 RA patients (70%) 68k specific T cells could be detected. Proliferation with PBMCs of healthy controls has been set as 100%. Stimulation is considered to be positive for values greater than 120% (p < 0.0001).

DETECTION OF 68K SPECIFIC T HELPER CELLS IN SYNOVIAL FLUID OF RA PATIENTS

To determine, if T cells primed for the 68k antigen were more abundant at the site of inflammation, synovial fluid derived T cells were also stimulated with the 68k antigen. Proliferation kinetics were non-uniform. From 11 RA patients tested, eight peaked on day 3 and three on day 10 (data of four patients shown in fig 1B). Those peaking on day 3 generally proliferated more vigorously compared with T cells from peripheral blood. Again, suppressor effects were observed upon blockade with anti-HLA II antibodies (data not shown).

**Discussion**

A 68k autoantigen had previously been shown to be a major target for self reactive antibodies in RA. Its sensitivity (66%) and specificity (99%) for RA are remarkable. In the present study autoreactive T cells specific for this antigen could be identified in 19 of 27 RA patients (70%). Although most of these patients were also anti-68k positive, this association did not reach statistical significance. The finding that a novel autoantigen is the target of highly disease specific antibodies and T cells emphasises the possible importance of the 68k antigen for initiation and maintenance of RA. It is now important to clone the antigen and to identify the relevant epitopes. In the case of the autoantibodies it has been shown to be a glycopeptide. These data were shown by deglycosylation (alkaline β elimination, enzymatically with O-glycosidase) and with sugar competition experiments (O-GlcNAc versus other sugars)(submitted data). The existence of the carbohydrate specific T cells is widely accepted now and it is challenging to test whether the 68k glycosidic epitope has a part to play for T cells as well.

Proliferation peaking on day 3 for SFMCs is an expected result when considering that the synovial joint is a primary site of antigenic stimulation and sensitisation, because so called primed T cells do not require the phase of induction to secrete interleukin 2 and express high affinity interleukin 2 receptors. Accordingly, the peak for mature T cells in response to a control antigen at day 5 is within the range for a recall response. The finding that the peak shifts to day 10 for PBMC and for some SFMC samples can be regarded as a mixed effect of T helper and T suppressive cells or maybe a result of a non-active disease state, or both. B cell proliferation is not likely to occur to a similar extent.

Inhibiting antigen presentation by blocking a certain HLA-allele product, for example HLA-DP, led to a decreased proliferation in some patients. This effect is to be expected when considering a regular T helper cell response restricted by the blocked HLA class II antigen. For other patients, however, a rise instead of a decrease in proliferation was seen. For these
patients we believe that by applying anti-HLA antibodies to inhibit suppressive T cells, and therefore counteracting the effect of the 68k specific helper T cells and thereby keeping the net incorporation rates low. The phenomenon is not unprecedented. For multiple sclerosis, leprosy, and type I diabetes, the existence of DQ restricted suppressor T cells is being considered.

Figure 3  (A) Increased 68k specific proliferation in the presence of anti-DP antibodies. Proliferation against the 68k antigen in the presence (DP) of a monoclonal antibody directed against HLA-DP is depicted for 27 RA patients. Seventeen of 27 RA patients (63%) present with an increased 68k specific proliferation when the HLA-DP antibody was added (proliferation in the presence of anti-DP antibodies + 68k antigen minus proliferation in the presence of the 68k antigen alone). (B) Increased 68k specific proliferation in the presence of anti-DQ antibodies. Proliferation against the 68k antigen in the presence (DQ) of a monoclonal antibody directed against HLA-DQ is depicted for 27 RA patients. Ten of 27 RA patients (37%) present with an increased 68k specific proliferation when the HLA-DQ antibody was added (proliferation in the presence of anti-DQ antibodies + 68k antigen minus proliferation in the presence of the 68k antigen alone). (C) Increased 68k specific proliferation in the presence of anti-DR antibodies. Proliferation against the 68k antigen in the presence (DR) of a monoclonal antibody directed against HLA-DR is depicted for 27 RA patients. Thirteen of 27 RA patients (48%) present with an increased 68k specific proliferation when the HLA-DR antibody was added (proliferation in the presence of anti-DR antibodies + 68k antigen minus proliferation in the presence of the 68k antigen alone).
suppressive T cells are solely restricted by DQ. Of even greater importance seem to be those that are restricted for DP and DR. At least in RA, antigen specific suppressive T cells are potentially restricted to any family member of HLA class II. This in turn may explain the still rather low incorporation rate after blocking one type of the presumptive suppressive T cells as most patients exhibited more than one type of suppressive T cells. Under these circumstances the unblocked subset could still exert its suppressive effect on the helper T cells. To prove the putative existence of 68k specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppressive T cells, they would have to be cloned and further analysed. Whether a specific suppression was responsible for the suppressive effects detected in this study, the specific antigen for cells secreting TGFβ must be present as a contamination in our experiments. This seems rather unlikely but can only completely be ruled out with the recombinant antigen.

Of interest is the finding by ourselves (unpublished results) and others,21,22 that the T2cytomegalovirus interleukin 10 could freely be detected in synovial fluids but not in the peripheral blood of RA patients. Although interleukin 10 is generally regarded as an inflammation suppressive cytokine, the donors suffered from active RA. Thus, it is speculated that certain down regulatory effects of interleukin 10 do not apply under RA conditions. In this respect, it was shown that an induction of class II molecules by GM-CSF or GM-CSF/interleukin 4 was no longer inhibited by interleukin 10 at up to 10 mg/ml.23 Increased interleukin 10 values were found together with increased monocine concentra-tions, so interleukin 10 may also lack an in vivo suppressive effect in monocyte activity.21

Why and how the 68k antigen becomes a target for autoreactive T cells and antibodies is not known. It is clearly demonstrated for this antigen that it is ubiquitously expressed and that autoantibodies directed against it could not be detected in healthy people. Thus, autoimmunity to the 68k antigen probably starts with some dysregulatory event in the immunological network, rather than with an overexpression or malexpression of the antigen. Whether or not cytokines such as interleukin 10, TGFβ or others are involved is under investigation. Nevertheless, an overexpression of the 68k antigen of certain cell populations cannot be excluded. Especially when glycopeptides are concerned, a slightly changed glycosylation pattern could result in autoimmunity remaining undetected with the antibodies used here.

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