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

St Bläß, C Haferkamp, Ch Specker, M Schwochau, M Schneider, E M Schneider

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.

(Ann Rheum Dis 1997;56:317–322)
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 stabilise 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 immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.

A 70k protein band showing no immuno-reactivity 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 antigen. A further control was NC without blotted protein. As estimated by Bradford quantification a 160 mm band contained approximately 0.2 µg of the 68k antigen.
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. 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 nor 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>
<th>DP</th>
<th>DQ</th>
<th>DR</th>
<th>DP/DR</th>
<th>DP/DQ</th>
<th>DQ/DR</th>
<th>DP/DQ/DR</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

**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-GlNAc 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.\textsuperscript{20–22} The data for RA presented in this paper do not support the idea that

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 patient shows one or other of the types of proliferative response will depend on many factors, such as an epitope spreading or a different HLA background, in common making up a private immune system. The mechanism by which suppressive cells could exert their antigen specific effect on the helper subset can so far only be speculated on. The two current models suggest either direct T to T cell contact or bystander suppression by TGFβ. It is noteworthy that if bystander 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,\textsuperscript{21,22} that the T\textsubscript{H}2 cytokine interleukin 10 could frequently 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.\textsuperscript{20} Increased interleukin 10 values were found together with increased monokine concentrations, so interleukin 10 may also lack an in vivo suppressive effect in monocytic activity.\textsuperscript{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 glycopeptid epitopes are concerned, a slightly changed glycosylation pattern could result in autoimmunity remaining undetected with the antibodies used here.

We thank Prof Dr G-R Burmester for critically reading the manuscript and helpful discussion. The technical assistance of Silke Mauelshagen is gratefully acknowledged. Supported by a grant from the Deutsche Forschungsgemeinschaft (Bu 445/4-1).

16. Werner P, Pawelec G, Schneider EM. Cellular detection of human class II antigens: Delimitation of a novel HLA-DP-like suppressor restriction system DR the sequential expression of class II antigens, and a pronounced functional flexibility of class II allogeneic reactive T cell clones. In: Solheim BG, Moller E, Ferrone S. HLA class II antigens. Berlin: Springer Verlag, 1986;81-98.
24. Schlak JP, Piers I, Meyer zum Buschenfelde KH, Hermann E. Detection of IL-2, IL-4, IL-6, IL-10, IFN-γ and TGF-β1 in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and miscellaneous other forms of arthritis. Clin Rheumatol 1995;14:236.
Rheumatoid arthritis: autoreactive T cells recognising a novel 68k autoantigen

St Bläß, C Haferkamp, Ch Specker, M Schwochau, M Schneider and E M Schneider

Ann Rheum Dis 1997 56: 317-322
doi: 10.1136/ard.56.5.317

Updated information and services can be found at:
http://ard.bmj.com/content/56/5/317

These include:

References
This article cites 23 articles, 6 of which you can access for free at:
http://ard.bmj.com/content/56/5/317#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

- Immunology (including allergy) (5144)
- Connective tissue disease (4253)
- Degenerative joint disease (4641)
- Musculoskeletal syndromes (4951)
- Rheumatoid arthritis (3258)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/