AUTOANTIBODIES AGAINST HUMAN CALPASTATIN IN RHEUMATOID ARTHRITIS: EPITOPE MAPPING AND ANALYSIS OF PATIENT SERA

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SUMMARY
Autoantibodies against calpastatin have recently been described to be highly prevalent in sera of patients with rheumatoid arthritis (RA). When the sera of 45 patients with RA were analysed for autoantibodies against calpastatin by a newly developed enzyme-linked immunosorbent assay (ELISA), only four sera (8.9%) tested positive, which is not significantly different from the frequency observed in healthy controls. Since the ELISA is based on a synthetic peptide containing the C-terminal 27 amino acids of calpastatin bound to the solid phase, this negative result might be the consequence of the small antigen used. Therefore, a systematic analysis of the epitopes for autoantibodies in calpastatin was performed using sera from RA patients and healthy individuals. Recombinant fusion proteins containing fragments of calpastatin or the complete protein were produced and sera analysed by Western blots. In the N-terminal portion (amino acids 1–369), at least two major epitopes exist, against which 65% of normal sera as well as 76% of RA sera show reactivity in Western blot assays. These epitopes are not useful for clinical diagnostics. Only five out of 45 (11.1%) RA sera reacted against the C-terminal portion (amino acids 363–708) of calpastatin, while four out of 52 (7.7%) control sera showed reactivity. Three of the five RA sera and two out of four control sera had autoantibodies against the C-terminal 27 amino acids of calpastatin. These three patient sera had already been tested positive in the ELISA. The fourth patient positive in the ELISA was Western blot negative. The differences between the group of RA patients and controls are not statistically significant. When the clinical characteristics of the four patients with autoantibodies against the carboxyl end of calpastatin were analysed, it became apparent that all four had significantly elevated C-reactive protein (>50 mg/l). This observation might indicate that calpastatin autoantibodies are found in RA patients with more active disease. Thus, while the majority of RA patients do not have an increased prevalence of calpastatin autoantibodies, it cannot be ruled out definitively that a small subgroup may be characterized by autoantibodies to the C-terminus of calpastatin.

KEY WORDS: Autoantibodies, Calpastatin, Rheumatoid arthritis, Epitope mapping.

CALPASTATIN (CAST) is the endogenous inhibitor of the intracellular, calcium-activated cysteine proteases calpain I and II (EC 3.4.22.17). These enzymes are ubiquitously expressed and show a wide substrate specificity in vitro. Even though our knowledge regarding their physiological role is far from comprehensive, limited proteolysis by calpains is implicated in numerous cellular regulatory processes. These include cytoskeletal rearrangements in the early phase of platelet aggregation and modulation of intracellular signalling cascades. Calpain is activated in response to increased calcium concentration, probably by autocatalytic processes and membrane translocation. Calpastatin is the most potent known inhibitor of calpain activity and is probably intimately involved in the regulation of the calpain system (for a review, see [1, 2]).

Interestingly, anti-calpastatin autoantibodies have been recently been identified by several groups independently [3–8]. The autoantibodies were detected in fertile patients [3, 4], in patients with rheumatoid arthritis (RA) [5, 6] and in patients with venous thrombosis [8]. One could hypothesize that autoantibody-mediated inhibition of the protease inhibitor calpastatin might lead to unbalanced activation of the proteases and subsequently to specific disease processes. Mimori et al. [5], in fact, could show an inhibitory effect of calpastatin autoantibodies on calpastatin in vitro. A very high prevalence of anti-calpastatin autoantibodies of 50% has been reported by two groups in RA patients [5, 6]. This observation could be of importance for an improved laboratory diagnosis of RA as well as for understanding the pathophysiology of the disease. The potential implications of anti-calpastatin autoantibodies in RA have been reviewed [9].

We also recently described the identification of anti-calpastatin autoantibodies, characterized the epitope and developed a specific peptide enzyme-linked immunosorbent assay (ELISA) based on the C-terminal 27 amino acids of calpastatin [7]. Our main focus was initially on patients with venous thrombosis because of the diagnosis of our first patient with anti-calpastatin autoantibodies. With the ELISA, a significantly elevated frequency of anti-calpastatin autoantibodies could be shown in patients with venous thrombotic events compared to normal controls [8].

Preliminary analysis of sera from RA patients using the ELISA revealed a low or only slightly increased prevalence of anti-calpastatin autoantibodies. Similar results were obtained by Western blot using a recombinant calpastatin fragment representing amino acids 612–708 of calpastatin. The simplest explanation for this apparent discrepancy would be that the calpastatin fragments used in our ELISA and Western blot procedures did not contain the relevant ‘RA epitope’. Such an epitope, if it existed, would be expected between
amino acids 531 and 612, because this sequence is common to the antigens used by the two other groups and not present in our peptide. Therefore, a thorough analysis of anti-calpastatin autoantibodies in RA patients and healthy probands was performed to address this issue.

**METHOD**

**Patients**

Sera from 45 patients (36 female, nine male) with RA were analysed in this study. Their mean age was 56.7 yr (s.d. 13.3 yr, range 28–84 yr). The patients were seen by one of us (BL) at the Rheumatic Disease Unit of the University Hospital of Regensburg. The diagnosis of RA was made if a patient fulfilled the American College of Rheumatology criteria for RA [10]. Rheumatoid factor was positive in 25 patients (55.6%; 18 female, seven male), and C-reactive protein was elevated (>8 mg/l) in 29 (64.4%; 23 female, six male). Sera of 205 healthy blood donors (113 male, 92 female) were collected and stored at −20°C. The mean age of this group was 26 yr (s.d. 6 yr, range 18–55 yr).

**Preparation of lymphocyte RNA**

Peripheral blood mononuclear cells were collected by means of a cell separator. These cells were further processed by centrifugation in an elutriator as pXa1 constructs. Transformants were grown at 37°C.

**Total RNA was isolated using the isothiocyanate**

Preparation of lymphocyte RNA

**Reverse transcription/polymerase chain reaction (RT/PCR)**

Total RNA was reverse transcribed by oligo dT priming, using a commercially available kit (Promega Corp., Madison, WI, USA) according to the manufacturer’s instructions. After reverse transcription, 50 pmol of the appropriate PCR primers and 3 mM MgCl₂ were added to a final volume of 50 μl. After the first denaturation step, 5 U Vent polymerase (New England Biolabs, Beverly, MA, USA) were added. The PCR primers were carried out in a Perkin-Elmer Cetus (Norwalk, CT, USA) thermocycler using the following protocol: 300 s 94°C, 35 × (44 s 92.3°C, 40 s 60.8°C, 46 s 71.5°C), 300 s 72°C. For amplification of the N-terminal part of calpastatin (amino acids 1–370), the oligonucleotides CAST163-BamHI (5’-ctagtcgacATCAATGAGTTCACAGGAATCCACAGAAACCAAGGC-3’) and CAST1273-SalI (5’-ctagtcgacATCAATGAGTTCAGTATCGACTCCG-3’) were used. Amplification of the C-terminal part of calpastatin (amino acids 188–708) was carried out with CAST724-BamHI (5’-ctagtcgacACCTACATAGGGAATTGGG-3’) and CAST2289-SalI (5’-ctagtcgacTCTAGATCTATTTTGCTTGGAAG-3‘). Further primers for amplification of CAST188–334 and CAST188–369 were CAST1164-SalI (5’-ctagtcgacCTATCCATATCCCAAGGG-3‘) and CAST1271-SalI (5’-ctagtcgacCTATCCATATCCCAAGGG-3‘). Further primers and 3′ ends for amplification of CAST1273-SalI (5’-ctagtcgacATCAATGAGTTCAGTATCGACTCCG-3’) and CAST1271-SalI (5’-ctagtcgacCTATCCATATCCCAAGGG-3‘) were CAST363–622 was amplified using primers CAST1249-BamHI (5’-ctagtcgacCTATCCATATCCCAAGGG-3‘) and CAST2028-SalI (5’-ctagtcgacCTATCCATATCCCAAGGG-3‘). All primers were obtained from MWG-Biotech GmbH, Ebersberg, Germany.

**Cloning, sequencing and expression**

RT/PCR amplified fragments were cut with the appropriate restriction enzymes and cloned into pUC18. Sequence analysis was performed as described earlier [7]. The full-length calpastatin cDNA was obtained by joining the two amino- and carboxy-terminal clones at a common SalI restriction site (position 1082). To obtain smaller partial calpastatin fragments, the respective parts were amplified from the pUC18 calpastatin clones with the appropriate primers. The PCR conditions were identical to those described for RT/PCR, except that the final MgCl₂ concentration was 2.5 mm and that 1 U of Taq polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) was used instead of Vent polymerase and added before the first denaturation step. For expression of the recombinant proteins, the inserts were subcloned into pXa1 [13]. Expression of the β-galactosidase fusion proteins was carried out as recently described [7]. In brief, Escherichia coli XLI蓝色 [14] were transformed with the recombinant pXa1 constructs. Transformants were grown at 37°C until the OD600 reached 0.4–0.6 and induced with 2 mm isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h.

**Prevalence of autoantibodies against recombinant calpastatin fragments in rheumatoid arthritis patients (RA) compared to healthy controls (NC)**

The numbers in the column ‘binding region’ indicate the smallest number of amino acids to which the autoreactivity of the sera tested can be narrowed. For instance, a serum positive against amino acids 1–370, but negative against amino acids 188–708 and 188–369, would fall into the category 1–370. No specific binding could be found between amino acids 188–334 and 612–682.

**TABLE I**

Prevalence of autoantibodies against recombinant calpastatin fragments in rheumatoid arthritis patients (RA) compared to healthy controls (NC). The numbers in the column ‘binding region’ indicate the smallest number of amino acids to which the autoreactivity of the sera tested can be narrowed. For instance, a serum positive against amino acids 1–370, but negative against amino acids 188–708 and 188–369, would fall into the category 1–188. No specific binding could be found between amino acids 188–334 and 612–682.

<table>
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<tr>
<th>Clones</th>
<th>Binding region</th>
<th>n (pos./all)</th>
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<td>33.3</td>
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<td></td>
<td>NC</td>
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<tr>
<td>(682–708)</td>
<td>NC</td>
<td>7/205</td>
<td>3.4</td>
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3–5 h. Cells were lysed and the crude extracts were analysed by 7.5% SDS–PAGE.

**Immunological assays**

Western blot analyses were performed as described [7]. Briefly, total protein of recombinant *E. coli* was separated by SDS–PAGE and semi-dry blotted on polyvinylidene fluoride (PVDF) membranes. After blocking with milk powder in phosphate-buffered saline, membranes were incubated with the sera in a dilution of 1:100. Bound IgG were detected using peroxidase-coupled anti-human IgG. For screening procedures, membranes were cut into strips (0.5 × 6 cm) and incubated in 2 ml diluted serum. Calpastatin-specific monoclonal antibodies CSF 1–2, CSL 1–5 and CSL 5–10 [15] (Dianova, Raboisen, Germany) were used for Western blot analyses at a concentration of 0.1 μg/ml. As a secondary antibody, an anti-mouse IgG conjugated to peroxidase (Dianova) was used at 0.8 μg/ml.

All patient and control sera were tested twice on each substrate. A serum was regarded positive if an immunoreactive band of the expected molecular weight was detectable. When the results were discordant for a given serum, the assay was repeated and the result of this third test was the one recorded. Positive reacting sera were tested for anti-β-galactosidase reactivity as described [7], to exclude non-specific binding to β-galactosidase.

The anti-calpastatin ELISA has been described in detail recently. Specifically reacting IgG against the C-terminal calpastatin fragment (peptide 682–708) can be detected and quantified with this assay. The results are expressed in arbitrary units. Values >30 U are considered positive [7]. The assay was provided by Progen Biotechnik GmbH, Heidelberg, Germany.

**RESULTS**

*Analysis of RA sera by ELISA*

When the sera of 45 RA patients were analysed by a previously described ELISA [7] for the presence of anti-calpastatin autoantibodies, only four sera (8.9%) were above the cut-off of the assay. This was not significantly different from the frequency of positive sera in healthy control persons, which is 3.4% [7]. Since the assay detects only antibodies against the C-terminal 27 amino acids of calpastatin, the presence of other potentially relevant epitopes in the calpastatin molecule was investigated.

**Isolation of a full-length calpastatin cDNA by RT/PCR**

Oligonucleotides were designed to amplify calpastatin from reverse-transcribed lymphocyte RNA. Two partial, overlapping calpastatin fragments were generated, and cloned into pUC18 for sequence verification. Amplification of the N-terminal part of calpastatin (amino acids 1–370) yielded a DNA fragment which lacked exon 3 (bases 184–249 coding for amino acids 8–29). Even though this splice variant has not been described in lymphocytes as yet, it has been detected previously in several cell types and tissues [16]. Otherwise, there were no differences in this fragment and the sequence deposited in the GDB databank (accession number D16217). The second clone with the C-terminal region (amino acids 188–708) was also amplified and sequenced. Its sequence was identical to the above-mentioned databank entry, except for a G1937A transition which would result in an amino acid exchange Glu592 to Gly. Furthermore, the 3′-untranslated end of calpastatin was 422 bp longer than expected. Both differences to the databank entry have previously been reported [5, 6]. The two calpastatin fragments were isolated from pUC18 and ligated at the SacI restriction site at position 1082, as described in Method, to obtain a full-length calpastatin cDNA which was again verified by sequence analysis.

**Expression of the recombinant proteins**

The calpastatin fragments (CAST1–370 and CAST188–708), whole calpastatin cDNA and PCR-generated subfragments were cloned into the expression vector pXa1, resulting in an overexpressed β-galactosidase fusion protein when cultured and induced in *E. coli* XL1 blue. A schematic overview of the expressed clones is shown in Fig. 1. In general, smaller fusion proteins were synthesized in larger amounts. Coomassie staining after SDS–PAGE of bacterial lysates with the overexpressed fusion proteins often showed additional smaller fragments besides the expected bands. This is probably due to incomplete synthesis or degradation of fusion proteins, as indicated by Western blots with monoclonal antibodies or calpastatin reactive sera (see below).

**Calpastatin fusion proteins stained in Western blotting**

In Fig. 1, the domain structure of calpastatin is shown and the relative position of the expressed calpastatin fragments. Figure 1 also illustrates the size of cDNA expression clones shown to react with sera from RA patients by Mimori et al. [5] and Despré et al. [6], both including the C-terminus (domains III and IV) of the protease inhibitor, with 178 and 284 amino acids, respectively. The initial clone found by our group (CAST612–708) is somewhat smaller (97 amino acids), but also contains the C-terminus. Expression of calpastatin or calpastatin fragments was demonstrated by Western blots with commercially available mouse monoclonal antibodies reactive against human calpastatin. As shown in Fig. 2, the monoclonal antibody (mAb) clone CSL 5–10 reacted with the whole calpastatin molecule (1–708), as expected, with CAST188–708 and with CAST363–622. No binding occurred to CAST1–370. The multiple bands observed in the Western blot are probably due to incomplete synthesis or degradation of the fusion protein, since it is never observed with sera binding the C-terminal epitope. The other two mAbs showed different binding sites. When the available information, which indicated that CSF 1–2 bound in calpastatin domain I, CSL 1–5 in domain III, and CSL 5–10 either in domain II or domain IV, was combined with
our own Western blot data, the binding regions of the three mAbs used could be narrowed to the following regions (see also Fig. 1): CSF 1–2 binds within amino acids 150–188 of calpastatin, CSL 1–5 binds within amino acids 427–563 and CSL 5–10 binds within amino acids 363–426.

Western blots with different calpastatin reactive sera are also shown in Fig. 2. The serum of patient H.M., in whom the initial calpastatin autoantibody was identified, binds to the C-terminal cDNA clone (CAST612–708), to whole calpastatin (1–708) and to the C-terminal part (CAST188–708), but shows only a weak reaction with the N-terminal clone CAST1–370 and no binding to the fragment CAST363–622. This is consistent with the data published earlier where the epitope of this patient’s calpastatin-reactive IgGs could be localized to the very C-terminus [7].

A further example demonstrates calpastatin-specific binding to at least two different epitopes: serum of a patient with systemic lupus erythematosus (SLE) (B6) recognizes the N-terminal fragment (CAST1–370) and CAST612–708. It should be noted that this serum also does not bind to peptide 363–622.

The Western blot results with an RA serum (RAR390) are shown as an example for a calpastatin-positive serum which does not recognize the C-terminal epitope. Typical are the double bands in the first two lanes from the left with whole calpastatin and CAST188–708. The lower bands represent incomplete or degraded fusion proteins, which apparently do not bind sera which are reactive against the C-terminus only (see above). These data clearly demonstrate that there is more than one epitope for autoantibodies within calpastatin.

**Western blot screening with sera from RA patients and normal controls**

Sera from patients with RA were analysed in order to obtain data on the prevalence of autoantibodies against calpastatin in these patients. The mean age of the RA patients was 56.7 yr, ranging from 28 to 84 yr. There were 36 women and nine men. Approximately 55% (25) tested positive for rheumatoid factor, and 29 had elevated C-reactive protein (>8 mg/l), indicative of active disease. Of these, 17 had C-reactive protein levels >20 mg/l and 11 had levels >50 mg/l.

In a first step, sera from 45 RA patients and 20 controls were tested for reactivity against β-galactosidase fusion proteins containing amino acids 1–370 (CAST1–370) or amino acids 188–708 (CAST188–708) (Fig. 3). Among the RA sera, 34/45 (75.6%) reacted against CAST1–370 and 20/45 (44.4%) reacted against CAST188–708. However, 13/20 (65.0%) control sera also reacted against CAST1–370 and 11/20 (55.0%) against CAST188–708, so that there was no significant difference between both groups (Table I).

Thereafter, the C-terminal half of calpastatin was analysed, since this region had been described previously as a highly prevalent autoantigen in RA patients [5, 6]. Two different calpastatin clones, namely CAST363–622 and CAST612–708 (initial clone), were used as antigens in Western blot assays with all 45 RA sera and 52 sera from healthy blood donors. A total
Fig. 2.—Western blots with different sera on several calpastatin fragments. Total protein of bacterial lysates with the overexpressed recombinant clones are separated by SDS-PAGE. The numbers in each lane indicate the respective amino acids of calpastatin in the expressed fusion protein (see Fig. 1). It should be noted that autoantibodies with reactivity against the C-terminal 27 amino acids, as in patient H.M., do not produce multiple bands with a lower molecular weight than the intact fusion protein as seen with antibodies with a more N-terminal binding site including the mAb CSL 5–10. This is also true for patient B6 who has an antibody against the C-terminal epitope confirmed by ELISA and an antibody to the N-terminal half of calpastatin. It is assumed that the additional bands represent incompletely synthesized or degraded fusion protein.

As expected from our previous data, all sera which bound to CAST612–708 also reacted with the very C-terminus (CAST682–708), indicating that these 27 amino acids contain the only epitope for RA sera within the C-terminal 100 amino acids of calpastatin. This means that more than half of the positive reacting sera against the C-terminal 346 amino acids [RA plus normal control (NC)] bind calpastatin within the C-terminal peptide used in the previously described anti-calpastatin ELISA (Table I). One serum that was positive in the ELISA was not detected by Western blot. This serum had only a slightly elevated titre (see of five RA sera (11.1%) and four normal control sera (7.7%) showed autoactivity against either one of the two autoantigens. Two RA and control sera each reacted against CAST363–622, while three RA sera and two control sera reacted against CAST612–708. None of the positive sera reacted against β-galactosidase alone.

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When the N-terminal part of calpastatin was analysed, a high prevalence of autoantibodies was detected both in RA and normal sera, as described above. Using subclones for the region between amino acids 1 and 370 (Fig. 1), it could be shown that ~42% (19/45) of RA sera recognized CAST1–188 (Table I). An
additional 15 sera (33.3%) were positive with CAST188–369, but not CAST188–334, in Western blot assays, indicating binding to amino acids 334–369. This binding pattern in domain II of calpastatin was also observed in patients with other underlying disorders (Fig. 4). The very high frequency of anti-calpastatin autoantibodies reactive against the N-terminal half of calpastatin in RA sera was similar to that observed in other studies using a C-terminal epitope. However, normal controls also showed a comparably high prevalence of anti-calpastatin autoantibodies: six out of 20 sera (30.0%) and seven out of 20 (35.0%) bound to the calpastatin regions 1–188 and 334–369, respectively. In summary, 34 out of 45 sera (75.6%) from RA patients and 13 out of 20 sera (65.0%) from normal controls reacted specifically with the N-terminal 370 amino acids of calpastatin. The difference between RA patients and normal controls is not significant, indicating that there is no disease specificity for these antibodies. At present, it is not clear why the N-terminal fusion proteins react with IgG from such a high percentage of sera obtained from apparently healthy persons.

Together with the previously described epitope (CAST682–708), which represents the peptide used for the anti-calpastatin ELISA, two narrow binding regions of IgG autoantibodies to calpastatin could be identified (Fig. 1): one between amino acids 334 and 369, and one at the carboxy-terminal 27 amino acids. Only the latter has been verified using a synthetic peptide (see also [7]). Furthermore, there are many sera with reactivity against amino acids 1–188. Unexpectedly, only very few sera bound specifically to amino acids 363–622.

**DISCUSSION**

Several groups have described autoantibodies against calpastatin in patients with different disorders. The most striking association of calpastatin antibodies with ~50% positive sera was reported independently by two groups in patients with RA [5, 6]. Both investigations were based on the detection of autoantibodies by Western blotting patient sera against recombinant calpastatin fusion proteins. In one study, the fusion protein contained amino acids 425–708 of calpastatin connected to glutathione-S-transferase [6], in the other amino acids 531–708 of calpastatin fused to β-galactosidase [5]. Des prés et al. [6] reported that none of 10 control sera reacted in the Western blot assay, while Mimori et al. [5] did not report any data on healthy controls. The very high frequency of anti-calpastatin autoantibodies in RA patients could not be reproduced in a preliminary analysis of our own RA patients using an ELISA based on a smaller antigen [7]. While the prevalence of autoantibodies against a fusion protein with amino acids 612–708 was <10% in RA sera, it was ~5% in control sera, indicating that calpastatin antibodies directed against the C-terminus of the protein are not more common in RA than in healthy controls.

This result indicated to us the possibility that the relevant epitope recognized by autoantibodies present in RA sera was different from the C-terminus of the protein. Such an epitope would be expected between amino acids 531 and 612, which represents the region of calpastatin contained in the antigens used by both other groups, but not in our initial antigen.

Analysis of sera from patients with RA did not support this assumption. Out of 45 sera from RA patients, only five (11.1%) showed reactivity against the C-terminal half of calpastatin (amino acids 363–708). Three of these sera (6.6%) reacted against the previously described C-terminal peptide (amino acids 612–708), while only two (4.5%) reacted against amino acids 363–622. These data indicate that the use of a larger antigen does not lead to a substantially higher prevalence of anti-calpastatin autoantibodies in our RA patients. In fact, the prevalence of autoantibodies binding between amino acids 363 and 622 is again similar in RA patients and healthy controls. Thus, the hypothesis of an ‘RA-specific’ epitope on calpastatin within the region from amino acid 531 to 612 could not be confirmed.

When the whole protein was screened for further major epitopes, two more antigenic regions could be identified. Autoantibodies against these two regions were found by Western blotting in high frequency in patient and control sera. It is unclear why normal sera have such a high prevalence of antibodies against the two N-terminal antigenic regions (amino acids 1–188 and amino acids 334–369). One explanation might be antigenic mimicry. However, data bank searches revealed no clue as to the nature of a potentially similar foreign antigen. Another possibility would be that there are natural autoantibodies against calpastatin.
This observation underlines the difficulties with screening patient sera in Western blot assays based on recombinant proteins. Depending on the structure of the recombinant proteins, there may be a high prevalence of positive sera in the general population. Furthermore, the interpretation of Western blot results is much more prone to investigator bias than, for instance, ELISA readings. Often, non-specific bands with a slightly different apparent molecular weight may be observed. Another problem is the fact that the time provided for signal development may profoundly influence the appearance of a blot. Therefore, a thorough analysis of control sera under the same experimental conditions is mandatory before any conclusions regarding disease associations can be drawn.

At present, the reason for the discrepancy between our data and the published results is not known. It cannot be ruled out that the fusion proteins used in the other trials might have some structural features which are not present in the fusion proteins containing amino acids 188–708 and 363–622 of calpastatin used in our analysis. However, such tertiary structure should be independent of the fused bacterial protein, because this was β-galactosidase in one study and glutathione-S-transferase in the other. In addition, the region between amino acids 425 and 530 should also have no influence on the tertiary structure relevant for antibody binding.

It is unlikely that the criteria for a positive result were more strict in our analysis, since we found more control sera to be positive than the two other groups. Finally, it cannot be ruled out that a subgroup of RA patients not sufficiently represented in our patients might have anti-calpastatin autoantibodies. Analysis of the four ELISA-positive patients revealed that all of them had C-reactive protein levels > 50 mg/l. In the absence of evidence for an active infection, this was interpreted as indicative of high disease activity. In fact, if the prevalence of anti-calpastatin autoantibodies is only regarded in the RA patients with elevated C-reactive protein, four out of 29 RA patients (13.8%) are positive. It is tempting to assume that the presence of anti-calpastatin autoantibodies is more likely in patients with active disease. However, the group of anti-calpastatin-positive RA patients in this investigation is clearly too small to make any general statement regarding the clinical course of their disease as compared to anti-calpastatin-negative RA patients. Analysis of more RA patients should resolve this question.

It is obvious that the structure of the antigens used, the definition of positivity in Western blot and the analysis of a sufficient number of healthy probands is critical for the interpretation of the data. The observation that antibodies against the N-terminal region of calpastatin are highly prevalent in healthy individuals makes it unlikely that any disease association will be found with these autoantibodies. On the other hand, the C-terminal 10–20 amino acids represent a well-defined epitope [7], and the prevalence of autoantibodies against this epitope has been found to be increased in thrombosis patients [8]. The availability of a specific ELISA to detect autoantibodies against this part of calpastatin will clearly facilitate the analysis of larger patient groups.

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