Antibodies against transglutaminases, peptidylarginine deiminase and citrulline in rheumatoid arthritis – new pathways to epitope spreading

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Abstract

Objective

The findings of the involvement of tissue transglutaminase (tTg) in the pathogenesis of coeliac disease (CD) have stimulated progress in the field of auto-immune diseases. Another calcium-dependent cysteine enzyme, peptidylarginine deiminase type 4 (PAD4), seems to be involved in the pathogenesis of rheumatoid arthritis (RA). There are obvious similarities between Tgs and PADs.

Methods

Using enzyme-linked immuno-sorbent assays, we have measured the occurrence of antibodies against guinea pig (gp) and human recombinant (hr) tTg, PAD and citrulline in 59 controls and 184 RA-patients, of whom 71 were treated with methotrexate (mtx).

Results

In addition to the expected antibodies against citrulline (62 %), sera from the 113 RA-patients without mtx treatment contained significantly increased frequencies of IgG anti-PAD (35 %), IgA anti-gp-tTg (34 %), IgA anti-hr tTg (20%), IgG anti-gp-tTg (13 %) and IgA anti-hr-FXIII (15%) compared to controls. In sera from the mtx-treated RA-patients the expression of antibodies was reduced. In patients not treated with methotrexate there was a statistically significant correlation between, on one hand, IgG anti-PAD and on the other hand, IgG anti-citrulline, IgA anti-gp-tTg, IgA anti-hr-tTg, IgG anti-gp-tTg, IgG anti-hr-tTg, or IgA anti-hr-FXIII. In the mtx-treated group these correlations were less pronounced.

Conclusion

In addition to the expected antibodies against citrulline, sera from RA-patients contained antibodies against PAD and against Tgs of at least two kinds, indicating that the specificity for anti-tTg in CD is far from complete. Most of the patients displayed more than one antibody, a possible indication of epitope spreading. MTX-treatment reduced the expression of antibodies.

Key words

Autoimmunity, citrulline, coeliac, epitope spreading, factor XIII, peptidylarginine deiminase, rheumatoid, transglutaminase, methotrexate.

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Introduction

Antibodies against peptide-bound citrulline are considered highly specific for rheumatoid arthritis (RA) (1). A family of enzymes, the peptidylarginine deiminases (PADs) (2), catalyzes the citrullination of arginine-containing peptides. The ureido group on the citrulline facilitates the unfolding of proteins due to decrease in net charge and loss of potential ionic bonds (3). Studies by Suzuki *et al*. (4) imply that the PAD4 haplotype is associated with susceptibility to RA. PAD4 is highly expressed in haematological cells and in RA synovial tissues. Deiminated α- and β-chains of fibrin have been pointed out as the major targets of RA-specific auto-antibodies (5).

In coeliac disease (CD), antibodies against endomysium (EMA) and gliadins are important complements of intestinal biopsies for establishing diagnosis. In 1997, Dieterich *et al*. (6) convincingly showed that tissue transglutaminase (tTg) is the major autoantigen of EMA. The normal Tg-catalyzed reaction is a transamidation between protein-bound glutamine and lysine residues, forming γ-glutamine-ε-lysine pseudo-peptide bridges between proteins, for example in the Factor XIII (FXIII) catalyzed stabilization of fibrin. In the absence of a potent nucleophilic second substrate, tTg can catalyze the hydrolysis of specific glutamine residues to glutamic acids in for example gliadins. This deamidation strengthens the recognition of gliadin by HLA-restricted gut-derived T-cells. For reviews, see (7, 8).

Antibodies against tTg have also been observed in conditions not directly connected to CD, such as inflammatory bowel diseases (9).

There are obvious similarities between Tgs and PADs. Both these post-translationally active families of thiol enzymes have similar molecular weights and are calcium-dependent, acting in the first step of the reaction by a nucleophilic attack on a polarized carbon atom in the substrate. In both cases, the products are ammonia and a peptide/protein with changed electrical charges and consequently with opportunities for new conformations. Furthermore, the expression of the subclasses of the two families of enzymes seems to be similar. Interestingly, the two types of enzymes might work sequentially. For example, maximal conversion of arginines to citrullines in a modified form of the hair follicle trichohyalin makes this structural protein a more efficient substrate for epidermal Tg (10).

Epitope spreading refers to a situation where immune responses develop to new epitopes, distinct from and non-cross reactive with the primary epitope causing the disease (11). The mechanisms for this phenomenon are still under investigation. Interestingly, in many cases enzymes and their substrates seem to be the auto-immune targets.

The strategy of the present work was to apply the progress made in understanding the pathogenesis of CD to the field of RA. Specifically, we wanted to evaluate the occurrence and correlation of antibodies against citrulline and the enzymes PAD, tTg and Factor XIII in sera from patients with RA.

Materials and methods

Patients and controls

All patients attending the Rheumatological Unit of Malmö University Hospital during 1995-2002, diagnosed with RA according to the criteria by the American College of Rheumatology 1987, were consecutively registered and systematically monitored. The median duration of disease at inclusion was 7 months (range 2-12). A total of 184 patients (53 males and 131 females; mean age (S.D.) 61.0 ± 14.0 years; range 22-84) were enrolled in the study. None had been diagnosed with CD, nor had they any signs or symptoms of CD. At the time of sampling, 71 patients had initiated treatment with methotrexate (MTX). Other treatments included chloroquine (n = 43), sulfasalazine (n = 13), gold sodium thiomalate (n = 2) and azathioprine (n = 1), while 54 patients received no treatment. Blood donors (30 males and 29 females; mean (S.D.) age 42 ± 16.2 years; range 19-65) served as controls.

All procedures were in accordance with the Ethical Committee of Lund University.

Assays of antibodies
For all determinations of antibodies, except anti-citrulline, cut-off was calculated as mean + 2 S.D. of controls. For IgG anti-citrulline the cut-off was defined according to the manufacturer's manual as > 25 units.
EMA were analyzed at the Department of Clinical Microbiology and Immunology of Lund University Hospital according to standard procedures.

Determination of PAD
For the analysis of IgG anti-PAD-antibodies, a rabbit skeletal muscle PAD (Sigma, batch 013K4128) was used as antigen. Microtitre plates (Maxisorp Nunc) were coated with 1 μg PAD/well, freshly prepared with 100 μl of 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) and 5.0 mM $CaCl_2$. After incubation overnight at 4°C, the plates were washed three times with washing solution (TBS with 10 mM EDTA and 0.1% Tween 20) and blocked for 30 minutes at room temperature (RT) with blocking solution (TBS with 0.5% Tween 20 and 1% human serum albumin (HSA)). Sera from patients with RA and healthy blood donors were diluted 200 times in washing solutions with 0.5% Tween 20. To each well 100 μl diluted serum was added in duplicate followed by incubation for one hour at RT. The washing procedure was repeated as above and 100 μl of peroxidase-conjugated antihuman IgG (DAKO), 1:5000 in washing solution, was added to each well. After incubation for one hour at RT the washing procedure was repeated once more and the plates were developed according to standard procedure with 1 mg OPD/ml, 0.1 M Na-citrate at pH 4.2 (DAKO), and 0.06% H_2O_2. The enzyme reaction was stopped after incubation at 30 minutes in darkness at RT by the addition of 100 μl 0.5 M H_2SO_4. Absorbance was estimated at 490 nm in a microplate-reader (E max). The intra-assay correlation of variation (CV) was 7.3 % (n = 8) and the inter-assay CV was 10.9 % (n = 3).

Determination of IgG anti-citrulline
The analysis of anti-CCP was performed in our laboratory with the Immunoscan RA Mark2 kit (Euro-Diagnostica) in accordance with the manufacturer's manual. As antigen, this method utilizes a synthetic, cyclic citrulline-containing peptide (CCP).

Determination of IgA and IgG anti-tTg antibodies
For the analysis of anti-tTg, a guinea pig liver Tg (gp-tTg, Sigma lot 122K7435; 1 μg/well) or a human recombinant Tg (hr-tTg, N-Zyme lot 0804T0021; 0.5 μg/well) was used. Freshly prepared solutions of the antigens in TBS containing 5.0 mM $CaCl_2$ were added to microtitre plates (CovaLink, Nunc). After incubation overnight at 4°C the plates were washed three times with washing solution (TBS with 10 mM EDTA and 0.1% Tween 20) and blocked for 30 minutes at RT with blocking solution (TBS with 0.5% Tween 20).
Sera from patients with RA and healthy blood donors were diluted 400 times (gp) or 1600 times (hr) with blocking solution, and 100 μl diluted serum were added to each well in duplicate followed by incubation for one hour at RT. The washing procedure was repeated as above and 100 μl of either peroxidase conjugated antihuman IgG (DAKO), 1:5000, or peroxidase conjugated antihuman IgA (DAKO), 1:1000 (gp) or 1:2000 (hr) diluted in washing solution, were added to each well. After incubation at RT for one hour the washing procedure was repeated once more. The plates were developed according to standard procedure with 1 mg OPD/ml, 0.1 M Na-citrate at pH 4.2 (DAKO), and 0.06% H_2O_2. After incubation at RT for one hour in darkness, the enzyme reaction was stopped by the addition of 100 μl 0.5 M H_2SO_4/well. Absorbance was estimated at 490 nm in a microplate-reader (E max).
The intra-assay CV of the guinea pig based method (n = 8) was 3.0% for IgA and 8.6% for IgG, while inter-assay CV was 6.1% for both IgA and IgG. The intra-assay CV of the method based on human recombinant tTg (n = 8) was 0.8 % for IgA and 1.3 % for IgG, while inter-assay CV was 3.1% for IgA and 7.1% for IgG.

Determination of IgA anti-hr-FXIII a subunit
For the analysis of antibodies against thrombin-activated FXIII, human recombinant FXIII (kindly supplied by Dr Bruce Carter, Zymogenetics), comprising the a subunit, was used as antigen. Microtitre plates (Maxisorp Nunc) were coated with 1 μg FXIII/well, freshly prepared with 100 μl of 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) including 5.0 mM $CaCl_2$ and 0.2 U human thrombin (Sigma, lot no. 21K 7602). The procedure was otherwise the same as for the analysis of antibodies against tTg. Intra-assay CV was determined as 3.9% (n = 8) and inter-assay CV was 1.7%.

Statistics
The statistical significance of differences was determined by the χ^2-test and 2-tailed Student's t-test. Due to skewed distribution of the values, logarithmic transformation was carried out before analysis. Spearman rank correlation was used to evaluate correlation. P-values < 0.05 were considered significant

Results
The levels of antibodies against CCP, PAD, tTg and FXIII in sera from the 184 patients with RA are presented in Figure 1. Of the 113 RA patients without MTX-treatment, 40 (35%) were positive for IgG anti-PAD, 70 (62%) for IgG anti-CCP, 38 (34%) for IgA anti-gp-tTg, 15 (13%) for IgG anti-gp-tTg, 22 (20%) for IgA anti-hr-tTg, 21 (19%) for IgG anti-hr-tTg and 17 (15%) for IgA anti-hr-FXIII. With the exception of IgG anti-hr-tTg, all antibodies were significantly elevated compared with the controls (Table I).
The corresponding figures for all the 184 patients, including the 71 MTX-treated were: 57 (31%) for IgG anti PAD, 109 (59%) for IgG anti-CCP, 55 (30%) IgA anti-gp-tTg, 22 (12%) for IgG anti-gp-tTg, 30 (16%) for IgA anti-hr-tTg, 24 (13%) for IgG anti-hr-tTg and 22 (12%) for IgA anti-hr-FXIII.
In sera from the 71 MTX-treated RA-patients only IgG anti-PAD, IgG anti-CCP and IgA anti-gp-tTg were statistically elevated (Table I).
When significantly elevated compared

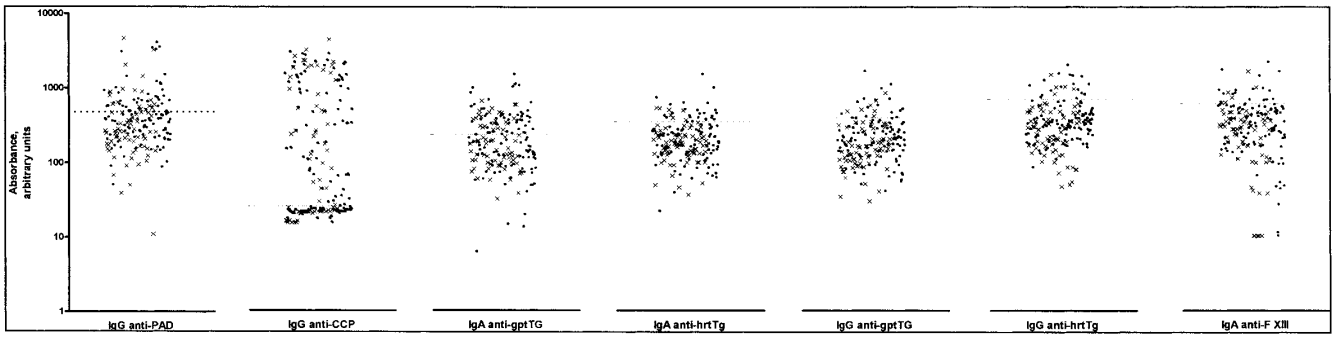

Fig. 1. The levels of antibodies in samples from RA-patients. •: not methotrexate treated, x: methotrexate treated. The horizontal lines indicate cut off levels.

Table I. Frequency (%) of positive antibody levels in the various groups of patients and controls. Statistical significance compared with the controls is indicated below the frequencies.

	All patients n = 184	Mtx-treated n = 71	Not mtx-treated n = 113	Blood donors n = 59
IgG anti-PAD	31 p < 0.0001	25 p < 0.0001	35 p < 0.0001	3.4
IgG anti-CCP	59 p < 0.0001	55 p < 0.0001	62 p < 0.0001	0.0
IgA anti-gp-tTg	30 p < 0.0001	25 p < 0.0001	34 p < 0.0001	0.0
IgG anti-gp-tTg	12 p = 0.023	9.9 p = 0.065	13 p = 0.011	3.4
IgA anti-hr-tTg	16 p = 0.080	9.9 p = 0.549	20 p = 0.026	6.9
IgG anti-hr-tTg	13 p = 0.204	7.0 p = 0.843	19 p = 0.085	6.1 (n=49)
IgA anti-hr-FXIII	12 p = 0.081	7.0 p = 0.573	15 p = 0.020	5.1

with the controls, the antibody levels of anti-tTg were not in any way as high as those found in true CD-patients (12) including infants (13). Of the 56 RA-patients who were positive for IgA anti-gp-tTg and the 28 RA-patient who were positive for IgA anti-hr-tTg, none were positive in the EMA test.

The various antibodies correlated markedly with each other. Especially, IgG anti-PAD correlated with all other antibodies and IgG anti-CCP correlated with all but IgG anti-hr-tTg and IgA anti-hr-FXIII. In contrast, IgG anti-gp-tTg did not correlate with most of the other transglutaminase antibodies. For details, see Table IIa.

The effects of treatment with MTX are illustrated in Figure 2. For all antibodies, patients treated with MTX displayed lower levels compared with patients on other treatment or with no treatment at all, with significant differences obtained for anti-PAD, IgA and IgG anti-hr-tTg, IgG anti-gp-tTg and IgA anti-hr-FXIII.

Discussion

Antibodies against CCP and PAD

Considering the situation in CD with antibodies against the substrate (gliadin) as well as against the corresponding enzyme (tTg), we found it worthwhile investigating the situation in RA with the enzyme/converted substrate pair, i.e. PAD and peptide bound citrulline. Our results, with a 59% frequency of IgG anti-CCP in sera from RA-patients, are in line with an earlier report (1). Moreover, we did not find any case of antibodies among the blood donors, further emphasizing the high specificity of IgG anti-CCP in RA. Interestingly, similar to a recent Finnish study (14), we also observed a significantly increased frequency of IgG anti-PAD among the RA patients. Furthermore, there was a high correlation between the prevalence of antibodies against PAD and CCP. Consequently, the situation in CD, with a combined immune reaction against an enzyme (tTg) as well as against a modified substrate (gliadin), is also observed in RA.

Antibodies against transglutaminases

In CD, the occurrence of serum IgA anti-tTg is regarded as a sensitive laboratory marker especially if the antigen is exposed to calcium during the coating in the ELISA procedure (13). In the present study on RA patients, 30% were positive for IgA anti-gp-tTg and 15% for IgA anti-hr-tTg. It should be emphasized that there were no signs or symptoms of CD among these RA patients. Furthermore, all patients with positive IgA anti-gp-tTg and anti-hr-tTg were EMA negative. IgA anti-hr-tTg has been observed in other intestinal disorders as well, such as ulcerative colitis and Crohn's disease (9). These patients were all EMA negative. In both the RA patients and in the patients with inflammatory bowel disease the tTg antibody titres were lower than those found in true CD patients, so it may be postulated that the tTg epitopes found in CD could be different from those found in RA. Compared with our data based on guinea pig liver Tg, Picarelli *et al*. (15), using native erythrocyte or recombinant human tTg as antigens, found a similar frequency of antibodies in RA. In contrast, Bizzaro *et al*. (16) reported a low frequency of anti-

Table IIa. The correlation (Spearman rank with 95% confidence interval) between antibodies in sera from the 113 RA-patients without methotrexate treatment.

	IgG anti-PAD	IgG anti-CCP	IgA anti-gp- tTg	IgG anti-gp- tTg	IgA anti-hr- tTg	IgG anti-hr- tTg
IgG anti-CCP	0.40 (0.23; 0.55) p < 0.0001	_____	_____	_____	_____	_____
IgA anti-gp-tTg	0.39 (0.22; 0.54) p < 0.0001	0.24 (0.05; 0.41) p = 0.01	_____	_____	_____	_____
IgG anti-gp-tTg	0.44 (0.28; 0.58) p < 0.0001	0.23 (0.04; 0.40) p = 0.02	0.39 (0.21; 0.54) p < 0.0001	_____	_____	_____
IgA anti-hr-tTg	0.25 (0.06; 0.42) p = 0.01	0.23 (0.04; 0.40) p = 0.02	0.56 (0.42; 0.68) p < 0.0001	0.13 (-0.07; 0.31) p = 0.18	_____	_____
IgG anti-hr-tTg	0.20 (0.01; 0.37) p = 0.04	0.03 (-0.16; 0.22) p = 0.75	0.09 (-0.11; 0.27) p = 0.37	0.13 (-0.06; 0.31) p = 0.17	0.28 (0.09; 0.45) p = 0.01	_____
IgA anti-hr-FXIII	0.36 (0.18; 0.52) p < 0.0001	0.18 (-0.01; 0.36) p = 0.05	0.63 (0.50; 0.73) p < 0.0001	0.15 (-0.04; 0.33) p = 0.11	0.52 (0.36; 0.65) p < 0.0001	0.15 (-0.04; 0.03) p = 0.10

Table IIb. The correlation (Spearman rank with 95% confidence interval) between antibodies in sera from the 71 RA-patients with methotrexate treatment.

	IgG anti-PAD	IgG anti-CCP	IgA anti-gp- tTg	IgG anti-gp- tTg	IgA anti-hr- tTg	IgG anti-hr- tTg
IgG anti-CCP	0.38 (0.15;0.56) p = 0.001	_____	_____	_____	_____	_____
IgA anti-gp-tTg	0.12 (-0.12;0.35) p = 0.32	0.09 (-0.15;0.33) p = 0.44	_____	_____	_____	_____
IgG anti-gp-tTg	0.20 (-0.04;0.42) p = 0.096	0.24 (0.00;0.46) p = 0.04	0.32 (0.09;0.52) p = 0.006	_____	_____	_____
IgA anti-hr-tTg	0.15 (-0.09;0.38) p = 0.21	0.29 (0.0.4;0.49) p = 0.02	0.35 (0.12;0.54) p = 0.003	0.04 (-0.20;0.28) p = 0.74	_____	_____
IgG anti-hr-tTg	0.29 (0.053;0.50) p = 0.01	-0.02 (-0.26;0.22) p = 0.86	0.02 (-0.22;0.26) p = 0.89	0.24 (0.00;0.46) p = 0.042	0.37 (0.15;0.56) p = 0.001	_____
IgA anti-hr-FXIII	0.09 (-0.16;0.32) p = 0.46	-0.01 (-0.25;0.23) p = 0.94	0.34 (0.11;0.54) p = 0.004	-0.22 (-0.44;0.02) p = 0.062	0.45 (0.24;0.62) P < 0.0001	0.07 (-0.17;0.30) P = 0.56

tTg in RA. Riente *et al*. also reported a low frequency of anti-tTg in RA both for anti-bovine and anti-human tTg antibodies (17).

Differences in the three-dimensional structure in bovine and guinea pig Tg may explain the varying results obtained for the two species, while the results for human tTg are more intriguing. The negative result obtained by Riente *et al*. was based on a commercial kit that has been used in several studies with good accuracy as regards CD. The varying results in RA may be due to differences in the tTg preparation or the technique used and/or a reaction towards other epitope(s). Some minor differences between the Eurospital kit and our method are most likely. Changes in, for example, the calcium concentration have great impact on the results for tTg antibody titres. Since the composition of the kit is not completely official it is not possible to fully explain the differences observed. Obviously, treatment with MTX might also contribute to this complex situation.

Thrombin-activated FXIIIa and tTg have similar characteristics and possibly also similar physiological functions. In an early preliminary study on RA synovial fluids, we detected Tg activity, sometimes originating from tTg and sometimes from Factor XIII, seemingly in an irreproducible pattern (18). The explanation was given many years later when we found that human monocytes express FXIIIa, but during the transformation of the cell, the ex-

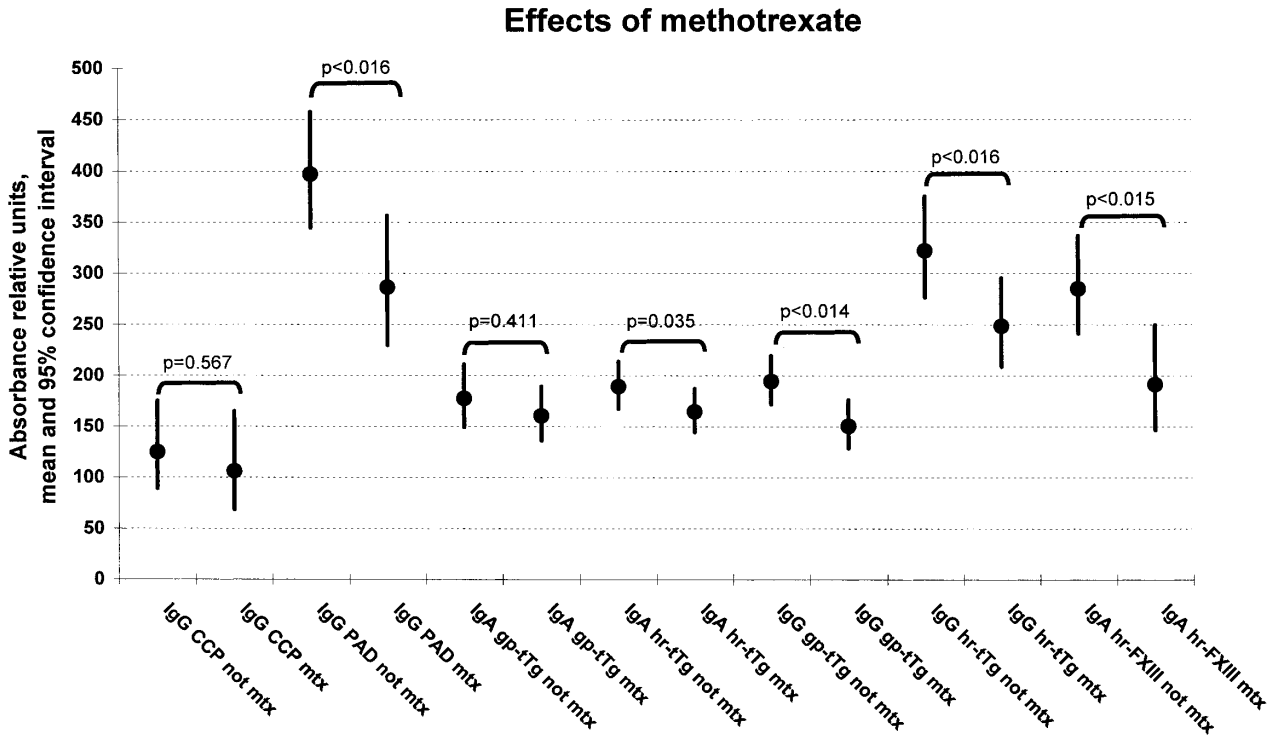

Fig. 2. A comparison between the relative levels of antibodies in samples from RA-patients with (mtx) and without (not mtx) methotrexate treatment.

pression of FXIII is down-regulated and substituted completely by tTg in the mature macrophage (19). Thus, although at a lower frequency, it is not surprising that we also observed IgA anti-FXIII in sera from RA-patients. This relationship is further emphasized by the high correlation between the occurrence of IgA against tTg and FXIII (Table IIa).

The effects of methotrexate

In general, patients treated with MTX had lower levels of antibodies than patients on no – or other – drugs. This result is in line with a recent prospective study that reported reduced levels of anti-tTg after treatment with MTX (15). In the case of MTX-treated patients, the correlations between the antibody levels were less clear, probably due to a general depression of antibody expression. Even though no immunosuppression, measured by a decrease in the total number of circulating leukocytes, occurred in a group of patients with SLE, their B-cells and different types of autoantibodies (ANA and anti-dsDNA among others) decreased after more than 10 weeks of MTX-treatment (20).

Epitope spreading

For the patients not being treated with MTX, there was a clear correlation between the levels of most antibodies. Compare the situation in CD, where antibodies against gliadins appear hand-in-hand with auto-antibodies against tTg. This intriguing situation may be explained by the formation of an immunogen complex between the substrate, gliadin, and the enzyme, tTg. The nature of this complex is still under investigation. *In vitro* in the presence of calcium and reducing agents, highly purified tTg forms high molecular weight structures with retained enzyme activity. Moreover, *in vitro* a substrate can be covalently incorporated into tTg via γ-glutamine-ε-lysine bonds. Indeed, a recent report showed that a complex between tTg and gliadin could be identified *ex vivo* (21). In 2003, we proposed another character of the complex, namely a thioester of the Michael's intermediate in the enzyme reaction (12). In the absence of a suitable nucleophilic substrate such as a primary amine, this thioester might be fairly long-lived. Recently, Fleckenstein *et al*. (22) were indirectly able to detect a thioester between human tTg and some synthetic, glutamine-containing gliadin peptides. They also found peptides incorporated into the enzyme via glutamyl-lysine pseudopeptide bridges. The reactions were performed *in vitro* in the presence of calcium and at pH 7.4. Obviously, at a lower pH such as in inflamed tissues, the fraction of the thioester might have been even higher. Indeed, this result strengthens our theory of a thioester being the antigen in CD (12).

Our present findings of antibodies in RA against the enzyme PAD and against the deiminated substrate, peptide-bound citrulline, resemble the situation with CD. It is probable that the PAD reaction occurs in three steps:

1. formation of the Michael's complex;
2. the formation of an amidino-enzyme intermediate with liberation of ammonia;
3. hydrolysis of the amidino-enzyme.

However, in contrast to the situation with tTg/gliadin, there are no obvious reasons to believe that peptide-bound arginine or citrulline residues will be incorporated into PAD. These facts favour the explanation of the covalent enzyme-substrate intermediate being the antigen.

If an enzyme such as tTg or PAD forms a temporary, covalent intermediate with a protein substrate previously modified by the enzyme, this complex might be antigenic, resulting in antibodies against both the enzyme and the modified substrate. This might explain the phenomenon called epitope (determinant) spreading. In such a situation, accidental activation of the enzyme plays the key role. Recently, we described that zinc at physiological levels inhibits the binding of CD antibodies to tTg (12). Indeed, zinc might be the physiological moderator of Tg activity. Unfortunately, with most enzymes including PAD, very little is known about the physiological moderation of enzyme activity.

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