

CONCISE COMMUNICATION

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Absence of citrulline-specific autoantibodies in animal models of autoimmunity

Autoantibodies directed to citrullinated proteins (e.g., anti-cyclic citrullinated peptide [anti-CCP]) (for review, see ref. 1) are highly specific markers of rheumatoid arthritis (RA) and can be detected in ~80% of patients with RA. These antibodies recognize proteins containing the nonstandard amino acid citrulline, which is the product of posttranslational modification of arginine residues by peptidylarginine deiminase (PAD) enzymes (for review, see ref. 2). The presence of citrullinated proteins in the synovium of patients with RA has been described previously (3). One of these citrullinated proteins was identified as fibrin (3), which is efficiently recognized by the anti-CCP antibodies. Recently, we described the presence of citrullinated proteins (including citrullinated fibrin) in the inflamed synovium of mice with collagen-induced arthritis or streptococcal cell wall-induced arthritis (4). The occurrence of synovial citrullinated proteins is therefore not specific for RA; instead, their presence appears to be associated with inflammatory processes. Although these mice express synovial citrullinated proteins, they do not produce citrulline-specific autoantibodies (4).

A recent report in *Arthritis & Rheumatism* (5) described the putative presence of anti-CCP antibodies in MRL-*lpr/lpr* and (NZW × B6)*F₁-hbcl-2*-transgenic mice that exhibited defects in regulation of lymphocyte apoptosis. MRL-*lpr/lpr* mice, which do not express the Fas antigen, develop an autoimmune syndrome similar to human systemic lupus erythematosus (6,7). The disease is characterized by the production of a large spectrum of autoantibodies, including anti-DNA and rheumatoid factor autoantibodies, and by the development of lymphadenopathy, glomerulonephritis, and arthritis. Similarly, (NZW × B6)*F₁-hbcl-2*-transgenic mice overexpressing human Bcl-2 in B cells also develop an autoimmune lupus-like disease but do not show signs of arthritis (Merino R: unpublished observations).

In the past, we investigated the presence of anti-CCP antibodies in numerous animal models of arthritis. Some animals indeed showed antibody reactivity against the citrullinated peptide cfc1-cyc (the CCP-1 peptide), but these animals also showed reactivity against the noncitrullinated control peptide cf0-cyc (Figure 1; for description of peptides, see ref. 8). The antibodies in these mice are therefore not citrulline-specific but rather are directed against other parts of the peptide. To investigate whether the antibodies detected in the MRL-*lpr/lpr* and (NZW × B6)*F₁-hbcl-2*-transgenic mice (5) were indeed citrulline-specific, as was suggested in this study, we measured antibody reactivity in these mice against the citrullinated cfc1-cyc and the noncitrullinated cf0-cyc peptides. Results, expressed as cutoff indexes (COIs), with the cutoff value defined as 3 SD above the mean value of control sera, are shown in Figure 1.

Sera of MRL-*lpr/lpr* mice reacted with both the citrullinated (median COI 3.4, interquartile range [IQR] 2.3–10.0) and the noncitrullinated peptide (median COI 5.6, IQR 2.7–10.7). There was a strong correlation between the degree of reactivity to both peptides ($r = 0.972$, $P < 0.0001$), indicating that the antibodies are directed to a part of the peptide other

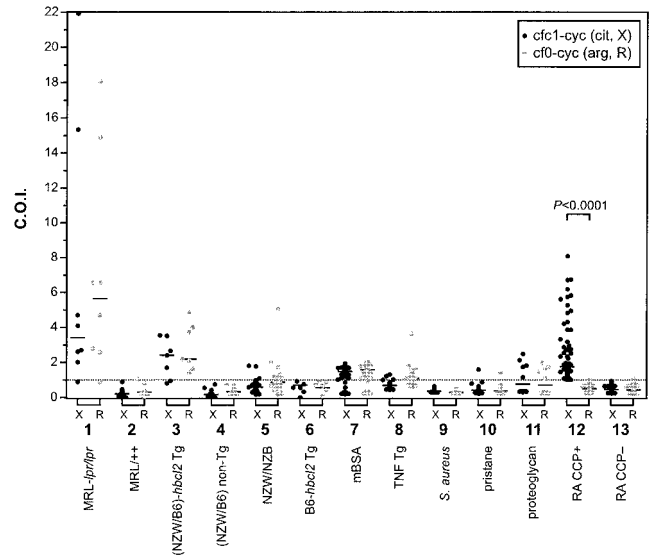


Figure 1. Serum levels of IgG antibodies to citrullinated peptide cfc1-cyc (solid circles, X lanes) and noncitrullinated control peptide cf0-cyc (shaded circles, R lanes) in various animal models of autoimmune disease and patients with rheumatoid arthritis (RA). Values are the cutoff indexes (COI), with the cutoff value defined as 3 SD above the mean value of control sera; solid horizontal lines show median values. 1 = 3-month-old MRL-*lpr/lpr* (n = 8 [described in ref. 5]); 2 = 8-month-old MRL/++ (n = 11 [described in ref. 5]); 3 = 7–8-month-old (NZW × B6)*F₁-hbcl-2*-transgenic (Tg) (n = 10 [described in ref. 5]); 4 = 7–8-month-old (NZW × B6)*F₁*-nontransgenic (n = 10 [described in ref. 5]); 5 = 8-month-old (NZW × NZB)*F₁* (n = 23 [described in ref. 5]); 6 = 8-month-old B6-*hbcl-2*-transgenic (n = 6 [described in ref. 5]); 7 = rabbits with methylated bovine serum albumin (mBSA)-induced arthritis (n = 57; kindly provided by Dr. Janet Dawson, Novartis Pharma, Basel, Switzerland [described in ref. 9]); 8 = tumor necrosis factor α (TNF α)-transgenic mice (n = 25; kindly provided by Dr. Günther Steiner, University of Vienna, Vienna, Austria [described in ref. 19]); 9 = mice with *Staphylococcus aureus*-induced arthritis (n = 17; kindly provided by Dr. Margareta Verdrehn, University of Göteborg, Göteborg, Sweden [described in ref. 20]); 10 = mice with pristane-induced arthritis (n = 23; kindly provided by Dr. Westley Reeves, University of Florida, Gainesville [described in ref. 21]); 11 = mice with proteoglycan (aggrecan)-induced arthritis (n = 10; kindly provided by Dr. Alison Finnegan, Rush University, Chicago, Illinois [described in ref. 22]); 12 = CCP1-positive RA patients (n = 65; in RA patients only, reactivity to the citrullinated peptide was significantly higher [$P < 0.0001$ by paired *t*-test] compared with the noncitrullinated control peptide); 13 = CCP1-negative RA patients (n = 117). Cit, X = citrulline; arg, R = arginine.

than the citrulline moiety (Figure 1, lanes 1X and R). Similar reactivity patterns were observed in (NZW × B6)*F₁-hbcl-2* mice (for cfc1-cyc, median COI 2.4, IQR 1.0–3.5; for cf0-cyc, median COI 2.2, IQR 1.6–4.0). In all of the other mice studied, no significant levels of antibodies to either the citrullinated or the noncitrullinated peptide were observed (median <1.0), despite the presence of active autoimmune disease in the

MRL/++ and (NZW × NZB)_F₁ mice. Only rabbits with methylated bovine serum albumin-induced arthritis (9) produced moderate levels of autoantibodies to peptides (for cfc1-cyc, median COI 1.5, IQR 1.1–1.7; for cf0-cyc, median COI 1.6, IQR 1.2–1.7) (Figure 1, lanes 7X and R). Again, however, the reactivity was not citrulline-specific.

We also measured antibody levels to both peptides in a selected group of 182 patients with RA. Sixty-five patients had a positive reaction with the citrullinated peptide (for cfc1-cyc, median COI 1.8, IQR 1.3–3.1), but none of the patients reacted with the noncitrullinated peptide (for cf0-cyc, median COI 0.5, IQR 0.4–0.6). Of the 117 patients who were nonreactive with the citrullinated peptide, only 1 was slightly reactive (COI 1.1) with the noncitrullinated peptide. These results are in concordance with our initial report on citrulline-specific autoantibodies in RA (10). At that time, it was shown that substitution of the citrulline residue by another amino acid (alanine, glutamic acid, glutamine, or ornithine) completely abolished reactivity with patient serum.

Our data thus show that citrulline-specific autoantibodies are present only in patients with RA and not in animal models of autoimmune disease. Many known autoantibodies are directed against proteins that become modified during cell death and in particular during apoptosis (for review, see refs. 11 and 12). When these modified self proteins are inefficiently cleared, they may be presented to the immune system and might not be recognized as self (13). In the presence of sufficient “danger signals” (as in an inflammatory environment), this can lead to an immune response against the modification (13,14). In most cases autoimmune patients also have generated autoantibodies to the unmodified antigen as a result of epitope spreading. In the case of citrullination, which is known to occur during terminal differentiation or cell death (15), the antibodies are directed only to the modification. This raises the question of what mechanisms are underlying this specificity. Some clues may be obtained from recent studies of HLA alleles. Several HLA alleles are known to be associated with RA, especially HLA-DR4 (HLA-DRB1*0401 and *0404) (16). Recently, it was shown that citrullinated peptides were able to bind to the HLA-DR4 shared epitope much more efficiently than were noncitrullinated peptides, and, in HLA-DR4-transgenic mice, T cells were readily activated by citrullinated antigens but not by unmodified antigens (17). Although there is no absolute requirement for HLA-DR4 in order to develop anti-CCP antibodies, there is a strong correlation between HLA-DR4 status and anti-CCP positivity in patients with RA (18). It thus seems likely that the genetic background is an important factor in the decision regarding whether citrulline-specific antibodies are produced (as in most RA patients) or not (as in the mouse models).

Although the production of cross-reactive antibodies to citrullinated cfc1-cyc and noncitrullinated cf0-cyc peptide in (NZW × B6)_F₁-*hbc1-2*-transgenic and MRL-*lpr/lpr* mice is clearly associated with alterations in the mechanisms that regulate B lymphocyte survival in these animals, the nature of the antigenic determinant recognized by these antibodies is unknown, because the animals produce antibodies to a multitude of antigens (modified or not).

Our results indicate that care should be taken when measuring antibodies to citrullinated proteins in animals. Reactivity to the noncitrullinated antigen should always be

measured as an essential control. In fact, this control appears to be also essential when using citrullinated proteins (e.g., citrullinated fibrinogen) instead of peptides for the detection of anticitrullinated protein antibodies in patients with RA, because circulating antibodies directed to the noncitrullinated protein may lead to false-positive results. This problem is less of a concern when using synthetic citrullinated peptides as the antigen, because peptides that give false-positive results (some peptides are reactive with human sera even when citrulline is replaced by arginine [Vossenaar E, et al: unpublished observations]) can be selectively left out. The peptides in the CCP assay have been optimized for not giving such false-positive results with human sera.

In conclusion, our present study demonstrates that autoantibodies to citrullinated antigens are highly specific for human patients with RA and have (thus far) not been observed in autoimmune animals. In addition, our results indicate that it is crucial to always use the corresponding noncitrullinated antigen as a negative control when surveying anticitrullinated protein antibodies.

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