RHEUMATOID ARTHRITIS

Comment on "*Aggregatibacter actinomycetemcomitans*–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis"

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The link between rheumatoid arthritis and exposure to a bacterial toxin was not found in a population of rheumatoid arthritis patients from Netherlands.

In a recent publication in *Science Translational Medicine*, Konig *et al.* (*1*) described a potential explanation for the link between periodontal infection and rheumatoid arthritis (RA). They identified a specific periodontitis-associated bacterium—*Aggregatibacter actinomycetemcomitans* (hereinafter *Aa*)—that can dysregulate the activity of citrullinating enzymes in neutrophils via its lytic toxin [leukotoxin A (LtxA)]. The generated citrullinated autoantigens are the target of a key autoantibody system in RA: anti-citrullinated protein antibodies (ACPAs). Furthermore, the authors report that the effect of the most important genetic risk factor for RA, the human leukocyte antigen–DRB1 shared epitope (HLA SE) alleles, was limited to RA patients who had been exposed to *Aa*, as determined by seropositivity to LtxA in a set of 194 RA patients. On the basis of these findings, the authors hypothesized that LtxA may be a key factor in the initiation of the RA-specific anti-citrullinated protein immune response in genetically predisposed individuals. In light of the crucial implications of this hypothesis for the current thinking regarding RA development, we aimed to replicate these findings.

To this end, we focused on two main questions: (i) Is the increased exposure to *Aa*, as measured by the presence of anti-LtxA antibodies, specific for RA or also present in other forms of inflammatory arthritis? (ii) Can we replicate the finding that the association between HLA SE alleles and ACPA-positive RA is limited to the anti-LtxA–positive subset?

To answer these questions, we established an enzyme-linked immunosorbent assay (ELISA) against purified LtxA. The purification of LtxA was performed according to the method described by Reinholdt *et al.* (*2*), which is the same method carried out from the same clone of the strain of *Aa* (HK921; JP2 positive) in the same laboratory as in the manuscript by Konig *et al.* (*1*). A hemolytic assay confirmed that the activity of LtxA corresponded to the normal range of freshly purified LtxA. To confirm that the presence of antibodies against LtxA was indeed a reflection of exposure to the bacterium *Aa*, we first tested serum samples from periodontitis patients (free from any chronic diseases such as RA) whose subgingival lesions had been tested for the presence of *Aa* by culturing techniques (*3*). As depicted in Fig. 1A, the concentrations of anti-LtxA antibodies were clearly increased in *Aa*-positive patients {median concentration [interquartile range (IQR)]: 8853 (5544; 14,147)} versus *Aa*-negative patients [median concentration (IQR): 764 (523; 4636)], confirming the specificity of the ELISA (Mann-Whitney *U* test, $P < 0.001$). Serial dilutions of a serum mix from three strongly positive RA patients were used as a standard, and the lowest point of the linear part of the standard curve (2000 arbitrary units/ml) was defined as the cutoff for positivity. With this cutoff, the specificity of our assay in *Aa*-negative periodontitis patients was 75%.

We then tested sera from 594 patients participating in the Leiden Early Arthritis Clinic with various different diagnoses, including RA according to the 1987 American College of Rheumatology criteria (*4*). Furthermore, we also measured anti-LtxA concentrations and positivity in a group of 156 healthy controls (without chronic illnesses) from the Leiden area. Figure 1B depicts the anti-LtxA concentrations in these groups and illustrates that anti-LtxA antibodies could be found in a substantial proportion of RA patients, as well as in patients with other forms of arthritis. The specificity of the assay in the healthy controls was 78.2%. Next, we investigated whether, among RA patients, anti-LtxA antibodies were preferentially present within the ACPA-positive group, and whether there was an association with the HLA SE alleles. As depicted in Fig. 1C, neither of these associations could be found in our cohort. Furthermore, as can be seen in Table 1,

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Table 1. The association of SE alleles with anti-CCP based on exposure to LtxA in patients with RA. Anti-LtxA, anti-leukotoxin antibodies as determined by enzyme-linked immunosorbent assay; anti-CCP, anti–cyclic citrullinated peptide antibody, cutoff for positivity >20 U; SE, HLA-DRB1 shared epitope allele; OR, odds ratio.

	Anti-LtxA–negative RA ($n = 143$)				Anti-LtxA–positive RA ($n = 189$)			
	SE-negative ($n = 44$)	SE-positive ($n = 99$)	OR	*P*	SE-negative ($n = 66$)	SE-positive ($n = 123$)	OR	*P*
Anti-CCP positivity (%)	34	70	4, 45	0.0001	29	68	5, 33	<0.0001

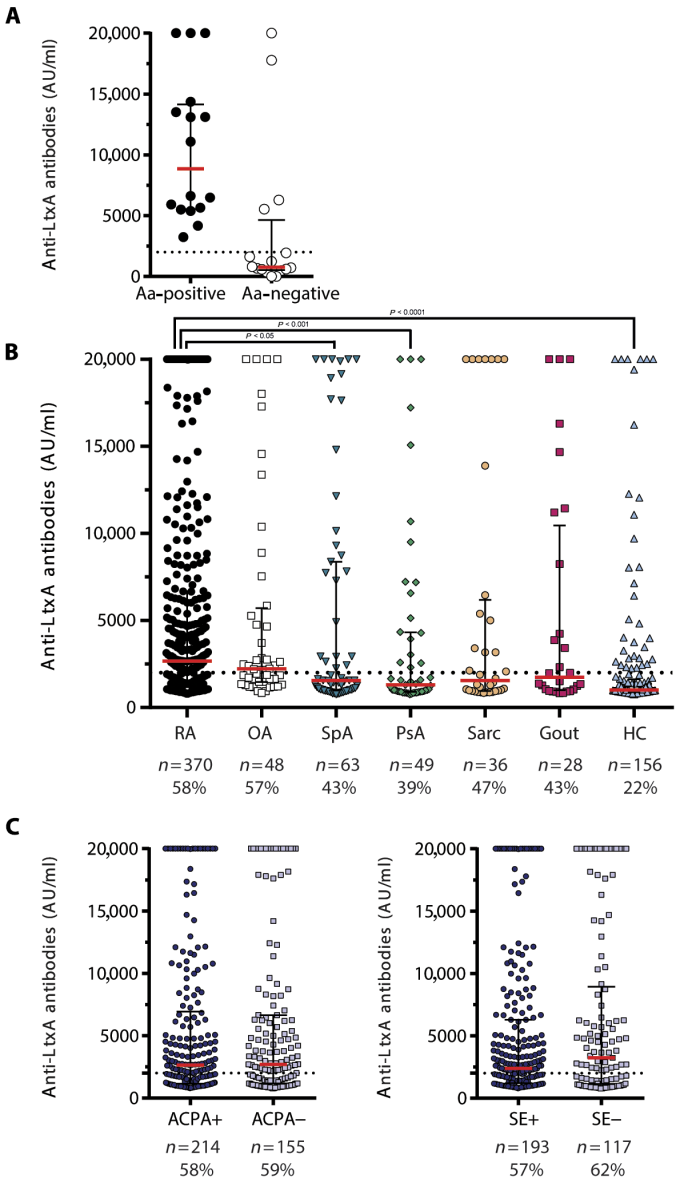

Fig. 1. Anti-LtxA antibody concentrations in various patient groups. (**A**) Serum antibodies to leukotoxin A (LtxA) were measured in periodontitis patients with and without *Aa* infection (n = 16 per group) confirmed by culturing. (**B**) Distribution of anti-LtxA antibodies in sera of 594 patients suffering from early arthritis and 156 controls. Concentrations of anti-LtxA antibodies in the serum of each individual are shown. (**C**) Serum antibodies to LtxA were measured in rheumatoid arthritis (RA) patients stratified for the presence of anti–cyclic citrullinated peptide 2 (CCP2) antibodies, classified as anti-citrullinated protein antibody (ACPA)–positive (n = 214) and ACPA-negative (n = 155) RA patients (left). Presence of anti-LtxA antibodies is shown in sera of RA patients carrying HLA-DRB1 shared epitope (SE) alleles, classified as SE-positive (n = 243) and SE-negative (n = 117) RA patients (right). OA, inflammatory osteoarthritis; SpA, spondyloarthritis with peripheral arthritis, PsA, psoriatic arthritis; Sarc, sarcoidosis. Red lines indicate the median for each group, and error bars indicate interquartile ranges. The dashed line indicates the cutoff for positivity. The number of patients per group and percentage of patients positive according to the cutoff are shown underneath. Mann-Whitney test was used for statistical comparison of antibody concentrations between (i) RA patients and other subgroups, (ii) ACPA-positive and ACPA-negative patients, and (iii) SE-positive and SE-negative patients. Significant differences are indicated by corresponding P values. AU, arbitrary units.

the association between HLA SE alleles and anti-CCP–positive RA was similar among RA patients positive and negative for anti-LtxA. Therefore, the effect of the HLA SE alleles appears not to be confined to the patient group positive for anti-LtxA antibodies.

In summary, in this large cohort of arthritis patients, anti-LtxA antibodies were not specifically associated with RA, and among RA patients, there was no association with the presence of ACPA or HLA SE alleles, in contrast to the findings of Konig *et al.* (*1*). A possible explanation for these divergent results could be that the anti-LtxA assay used here may have differed from the assay in the previous publication. However, the essential constituent (LtxA) and setup of the assay were derived from the same source. Furthermore, we performed various controls, such as analysis of periodontitis patients, the results of which support the validity of our assay.

It is also possible that differences in proportions of positive patients reported in the original study by Konig *et al.* (*1*) and in our study could be due to differences in patient populations between the United States and Netherlands. It seems likely that differences in living environment, genetic background, and referral strategy exist between these two countries. However, because *Aa* is a prevalent microorganism causing periodontitis in both countries (*5*, *6*), it appears unlikely that population differences can explain the contrasting findings regarding the possible role of *Aa*.

Although microbial influences may well be important in the development of RA, our results do not support a key role of exposure to LtxA originating from the periodontal pathogen *Aa* in linking the effect of the HLA SE alleles and periodontal disease to anti-citrullinated protein autoimmunity in RA. The hypothesis of a causal link between periodontitis/periodontitis-associated microorganisms and RA seems appealing, but it is also possible that RA patients are more frequently anti-LtxA–positive simply because they have more periodontitis, as is known from epidemiological studies (*7*, *8*). We hope that our findings can contribute to the discussion about the origins of autoimmunity in RA, and look forward to other replication experiments regarding these intriguing observations.

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