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Association of Smoking With the Constitution of the Anti–Cyclic Citrullinated Peptide Response in the Absence of HLA–DRB1 Shared Epitope Alleles

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Objective. **Smoking is a risk factor for anti–cyclic citrullinated peptide (anti-CCP) antibody–positive rheumatoid arthritis (RA) in patients with HLA–DRB1 shared epitope (SE) alleles. It is unknown whether smoking influences not only the presence of these antibodies, but also other characteristics of the anti-CCP response, such as isotype usage. The aim of this study was to determine the influence of smoking on anti-CCP isotypes in RA patients, and to determine whether this influence is observed in the presence and/or absence of SE alleles.**

Methods. **IgA, IgM, and IgG subclasses of anti-CCP antibodies were measured by enzyme-linked immunosorbent assay in serum obtained at the first visit to the Leiden Early Arthritis Clinic from 216 patients with anti-CCP–positive RA whose smoking habits were also assessed. HLA genotyping data were available for 202 of these patients.**

Results. **IgA and IgM anti-CCP were more frequent in RA patients who were smokers than in those who were nonsmokers (odds ratio 2.8 and 1.8, respectively). In addition, levels of all isotypes of anti-CCP, except IgG3, were significantly higher ($P < 0.05$) in smokers. The number of anti-CCP isotypes was higher in smokers compared with nonsmokers, both in SE-negative RA ($P = 0.04$) and in SE-positive RA ($P = 0.07$).**

Conclusion. **Patients with anti-CCP–positive RA who have a current or former tobacco exposure display a more extensive anti-CCP isotype usage in general, and IgA and IgM in particular, compared with patients with anti-CCP–positive RA who have never smoked. In contrast to its influence on the incidence of anti-CCP positivity, the influence of tobacco exposure on the constitution of the anti-CCP response is significant in SE-negative RA. These findings suggest a differential effect of tobacco exposure on the induction as compared with the propagation of the anti-CCP antibody response.**

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Antibodies against citrullinated proteins are thought to play a pivotal role in the progression of rheumatoid arthritis (RA) because they are highly specific and predictive of RA (1), are associated with the extent of joint destruction (2), and have been shown to enhance disease severity in mice with experimental arthritis (3). The most prominent genetic risk factors for RA, the HLA–DRB1 shared epitope (SE) alleles, encode for a common amino acid sequence in the peptide presenting part of the HLA class II molecule. These SE alleles have been described recently to be a risk factor for the development of anti–cyclic citrullinated peptide (anti-CCP) antibodies, rather than for anti-CCP–positive RA per se (4).

Smoking is a well-known environmental risk factor for the development of RA (5) and has been reported to influence the severity of RA in terms of

disease expression, disease activity, and radiologic joint damage (6,7). However, tobacco exposure has been associated with anti-CCP–positive RA only, as opposed to RA in general. This association was only observed in the context of SE alleles, and not with SE-negative RA, thus demonstrating a gene–environment interaction between the HLA–SE and smoking (8,9). Together, these observations were the basis for the hypothesis, first postulated by Klareskog et al (9), that smoking may trigger RA-specific immune reactions to citrullinated proteins, possibly by inducing citrullination of damaged, dying cells in the bronchoalveolar tract.

The observation that the gene–environment interaction between HLA–SE alleles and smoking was only present in anti-CCP–positive disease (9) makes it attractive to speculate that smoking may affect not only the presence, but also the "nature" of the anti-CCP response. For example, it is conceivable that the contribution of HLA–SE alleles to the association between smoking and anti-CCP–positive disease is routed through CD4+ T helper cells, which influence the magnitude and/or quality of the citrullinated protein–directed B cell responses and thereby the overall anti-CCP response.

We have recently shown that the levels of anti-CCP antibodies in patients with SE-positive, anti-CCP–positive arthritis who smoked were higher compared with those in patients with SE-positive, anti-CCP–positive arthritis who never smoked (10). However, no information is available on the constitution of the anti-CCP response with respect to, for example, isotype usage as a characteristic of the anti-CCP response. This information could be of relevance because it might provide novel details on the relationship between anti-CCP antibodies, the HLA–SE, and smoking in patients with RA, and subsequently may increase the understanding of how tobacco exposure contributes to the development and progression of RA.

Smoking is associated with a higher prevalence of citrullinated proteins in cells obtained by bronchoalveolar lavage (9), presumably caused by abundant protein citrullination in damaged cells. Therefore, the effect of smoking on the anti-CCP response could be mediated through modulation of citrulline-directed immune responses in the bronchus-associated lymphoid tissue (BALT). We hypothesized that the prevalence of IgA anti-CCP, and possibly other isotypes, would differ between RA patients with and those without tobacco exposure, since IgA is an isotype that is typically, although not exclusively, produced in mucosa-associated lymphoid tissue such as BALT.

Levels of total IgG anti-CCP are commonly measured in studies and in daily clinical practice. However, little information on the IgA, IgM, and subclasses of IgG anti-CCP antibodies is available, in contrast to the extensive study findings on the isotypes used by rheumatoid factor (RF)–producing B cells. IgA-RF has been reported to be associated with a more severe disease outcome, and smokers have been reported to produce more IgM-RF and IgA-RF as compared with the levels of these isotypes in nonsmokers (7).

In this study we first addressed whether the isotype usage in patients with anti-CCP–positive RA who were smokers differed from the isotype usage in patients with anti-CCP–positive RA who were nonsmokers, focusing especially on the participation of IgA in the anti-CCP response. We then analyzed whether this influence of tobacco exposure on isotype usage depended on the presence of HLA–DRB1 SE alleles, as was recently described with respect to the influence of tobacco exposure on the presence of IgG anti-CCP antibodies in RA patients. Our data demonstrate that smoking influences the pattern of isotype usage in the anti-CCP response, and that this effect is not limited to SE-positive RA.

PATIENTS AND METHODS

Study population and serum samples. Patients having received a diagnosis of RA within the first year after their initial clinic visit were selected from the Leiden Early Arthritis Clinic (EAC), which provides an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). The EAC was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and is described in detail by van Aken et al (11). RA was diagnosed according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA (12).

At the first EAC visit, serum samples were obtained and smoking history (all sorts of active tobacco exposure) was assessed by means of patient questionnaires. Patients who were current smokers and those with a history of smoking were classified as smokers, while those who had never smoked were classified as nonsmokers. Patients for whom baseline serum samples and smoking history were available were selected for the present study ($n = 416$). Anti-CCP antibody isotypes were assessed in IgG anti-CCP–positive patients only, resulting in a cohort of 216 patients for inclusion in the present study. Among the 216 patients with anti-CCP–positive RA, 202 had data available on the HLA genotype. Patients provided their informed consent, and the study was approved by the local review board of medical ethics.

Isotypes of anti-CCP antibodies. Total IgG anti-CCP antibodies were assessed by an enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Euro-Diagnostica,

Table 1. Distribution of isotypes of anti-CCP in 117 smokers versus 99 nonsmokers*

Anti-CCP isotype	Nonsmokers, no. (%)	Smokers, no. (%)	Odds ratio (95% CI)
IgA	50 (51)	87 (74)	2.8 (1.60–5.04)
IgM	55 (56)	81 (69)	1.8 (1.03–3.15)
IgG1	99 (100)	116 (99)	–
IgG2	76 (77)	99 (85)	1.7 (0.84–3.30)
IgG3	50 (51)	70 (60)	1.5 (0.85–2.51)
IgG4	97 (98)	113 (97)	0.6 (0.10–3.25)

* Anti-CCP = anti–cyclic citrullinated peptide; 95% CI = 95% confidence interval.

Arnhem, The Netherlands). The cutoff for IgG anti-CCP positivity was set at a level of 25 units/ml, according to the manufacturer's instructions.

Levels of the IgG subclasses of anti-CCP as well as levels of IgA and IgM anti-CCP were determined by a sandwich ELISA technique as described previously (13). Briefly, microtiter plates coated with CCPs (Immunoscan RA Mark 2; Euro-Diagnostica) were incubated with the patients' serum. The next incubation step was performed with conjugated polyclonal antibodies for the detection of IgM and IgA (AHI 0605 and AHI 0105; BioSource International, Camarillo, CA), and unconjugated mouse monoclonal antibodies followed by conjugated rabbit anti-mouse Ig for the detection of the IgG subclasses. A series of successive dilutions of pooled patient sera was used as a reference standard in all plates. Microtiter plates coated with uncitrullinated control peptide (Euro-Diagnostica) were used as a control for citrulline specificity of the antibodies.

Cutoff values for the presence of the different isotypes of anti-CCP antibodies were defined as the mean plus 2 SD in serum samples of a group of 50 IgG anti-CCP–negative control subjects, and were corrected for a high background level of response against the control peptide, as described previously (13). The cutoff values for positivity were as follows: 25 units/ml for IgA anti-CCP, 32 units/ml for IgM anti-CCP, 2 units/ml for IgG1 anti-CCP, 20 units/ml for IgG2 anti-CCP, 52 units/ml for IgG3 anti-CCP, and 0.1 units/ml for IgG4 anti-CCP.

HLA genotyping. The HLA–DRB1 alleles were determined in 202 patients with anti-CCP–positive RA. HLA–DRB1 (sub)typing was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides as previously described (14). The SE alleles were DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *1001, and *1402.

Statistical analysis. Odds ratios (ORs) were calculated by comparing patients whose serum was positive and patients whose serum was negative for the different anti-CCP isotypes. Differences in levels of anti-CCP isotypes and differences in the number of anti-CCP isotypes were analyzed using the Mann-Whitney U test. SPSS software, version 12.0 (SPSS, Chicago, IL) was used for all statistical analyses. In all tests, P values less than 0.05 were considered significant.

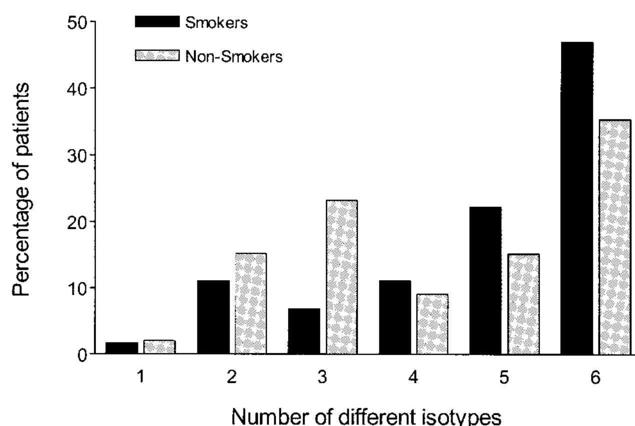

Figure 1. Percentage of patients with a certain total number (per patient) of each anti–cyclic citrullinated peptide (anti-CCP) isotype, among patients with IgG anti-CCP–positive rheumatoid arthritis who were classified as smokers (n = 117) or nonsmokers (n = 99).

RESULTS

Different classes and subclasses of anti-CCP antibodies were determined in 216 patients with anti-CCP–positive RA to determine whether tobacco exposure influences the usage of the different isotypes, and in particular the presence of IgA anti-CCP. We found that IgA anti-CCP was more frequently present in smokers than in nonsmokers, with an OR of 2.8 (95% confidence interval [95% CI] 1.60–5.04). IgM anti-CCP was also more frequent in smokers than in nonsmokers (OR 1.8, 95% CI 1.03–3.15), whereas the subclasses of IgG anti-CCP were not significantly more frequent among smokers (Table 1).

A trend toward longer disease duration at the time of inclusion was observed for the patients classified as smokers compared with those classified as nonsmokers (P = 0.08 by Mann-Whitney U test), and therefore additional logistic regression analyses were performed to correct for disease duration. Smoking was still found to

Table 2. Levels of anti-CCP isotypes in smokers versus nonsmokers*

Anti-CCP isotype	Anti-CCP isotype level, units/ml		P†
	Nonsmokers	Smokers	
IgA	67 (42–141)	109 (47–352)	0.012
IgM	57 (42–98)	94 (52–166)	0.001
IgG1	145 (79–208)	201 (108–312)	0.003
IgG2	71 (40–167)	125 (57–328)	0.016
IgG3	145 (72–266)	186 (86–870)	0.102
IgG4	17 (5–63)	75 (15–363)	<0.001

* Values are the median (interquartile range). Anti-CCP = anti–cyclic citrullinated peptide.
† Calculated by Mann-Whitney U test.

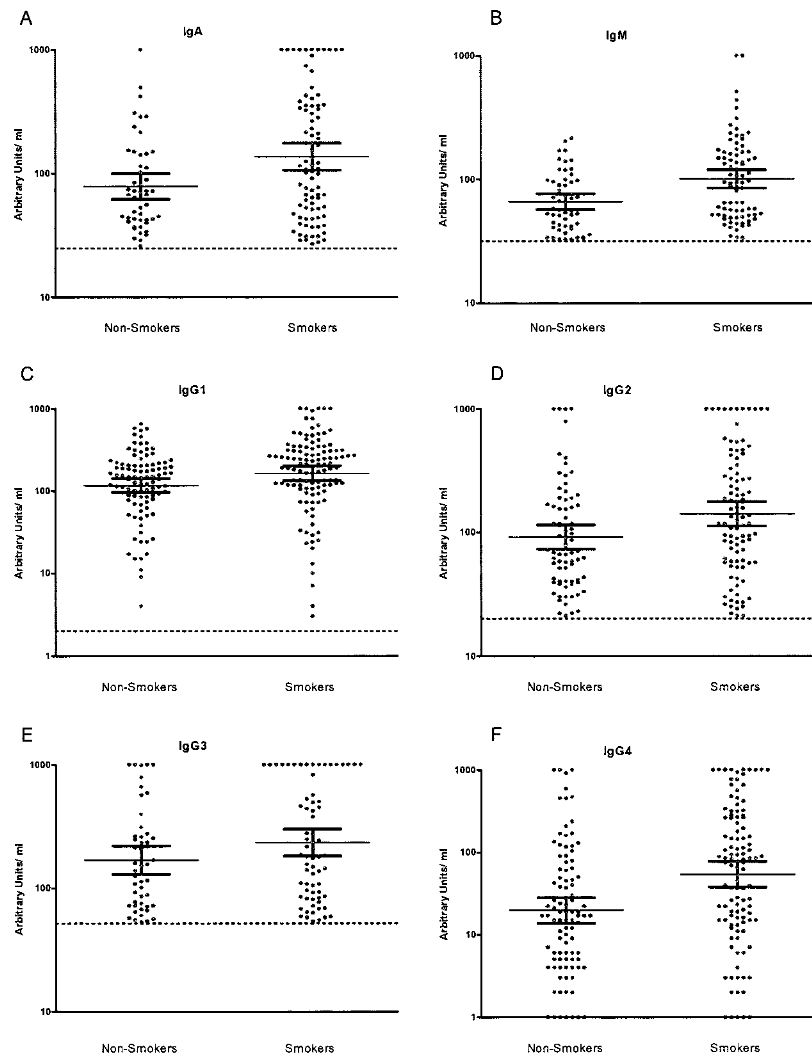

Figure 2. Levels of IgA anti–cyclic citrullinated peptide (anti-CCP) (**A**), IgM anti-CCP (**B**), IgG1 anti-CCP (**C**), IgG2 anti-CCP (**D**), IgG3 anti-CCP (**E**), and IgG4 anti-CCP (**F**) in patients with anti-CCP–positive rheumatoid arthritis who were positive for the respective isotypes and classified as nonsmokers or smokers. Circles indicate individual data points. Broken lines indicate the cutoff level for positivity. Bars show the geometric mean with 95% confidence interval.

be a significant predictor of both the presence of IgM ($P = 0.022$) and the presence of IgA ($P = 0.001$) after correction for disease duration.

To summarize the extensiveness of the isotype usage, the number of different isotypes participating in the anti-CCP response in individual patients was calculated. Although the median number of isotypes used was equal between patients who were smokers and those who were nonsmokers (median 5 isotypes, range 1–6), the number of isotypes detected per patient was higher in smokers compared with nonsmokers ($P = 0.013$ by Mann-Whitney U test) (Figure 1), indicating that tobacco exposure influences the extensiveness of anti-CCP antibody isotype usage in general, and of IgM anti-CCP and IgA anti-CCP in particular.

To determine whether tobacco exposure influences not only the presence or absence of the different isotypes of anti-CCP antibodies, but also the level of each isotype, the different anti-CCP isotypes were measured by ELISA in the serum, and levels were compared

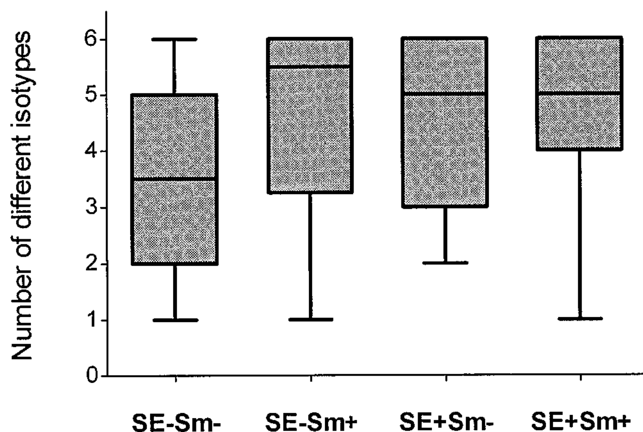

Figure 3. Distribution of the median number of different anti–cyclic citrullinated peptide (anti-CCP) antibody isotypes in patients with anti-CCP–positive rheumatoid arthritis who were classified as nonsmokers (Sm−) or smokers (Sm+) in the presence (SE+) or absence (SE−) of HLA–DRB1 shared epitope alleles. Results are shown as box plots, where the bars indicate the median, boxes indicate the first and third quartiles, and bars outside the boxes indicate the range. P = 0.04 for SE−Sm− (n = 20) versus SE−Sm+ (n = 16); P = 0.07 for SE+Sm− (n = 70) versus SE+Sm+ (n = 96), by Mann-Whitney U test.

according to tobacco exposure in RA patients who were positive for the respective anti-CCP isotypes. Levels of all isotypes of anti-CCP antibodies, except those of IgG3, were significantly higher in the patients classified as smokers than in the patients who had never smoked (Table 2 and Figure 2), which is consistent with previous results with regard to total levels of IgG anti-CCP antibodies (10).

We then assessed whether the influence of tobacco exposure on isotype usage can be observed in both SE-positive and SE-negative RA, and whether the influence of smoking is dependent on the presence of SE alleles in RA, as was recently described with respect to the influence of tobacco exposure on the presence of anti-CCP antibodies. In the present analysis, patients were stratified according to tobacco exposure and the presence or absence of SE alleles.

IgA anti-CCP, irrespective of SE status, was significantly more frequent among smokers. Similarly, IgM anti-CCP was more often detected in smokers as compared with nonsmokers regardless of whether these patients had SE-positive or SE-negative disease, although the differences were not statistically significant (data not shown). A trend toward a higher number of different isotypes of anti-CCP antibodies in smokers compared with nonsmokers was observed in those with SE-positive RA (P = 0.07).

More intriguingly, however, we observed that in the patients with SE-negative RA, tobacco exposure was associated with a more extensive isotype usage within the anti-CCP response (P = 0.04) (Figure 3). No interaction between SE status and smoking status in relation to usage of the anti-CCP antibody isotypes could be detected (data not shown). However, the data obtained indicated that the influence of smoking on isotype usage in patients with anti-CCP–positive RA does not depend on the presence of SE alleles.

DISCUSSION

B cells activated in the bronchoalveolar tract are prominent producers of IgA antibodies, and the organized BALT that is involved in the generation of IgA-producing cells can be detected more frequently in smokers than in nonsmokers (15). This finding, together with the observation that individuals who are smokers display higher citrullination in cells obtained by bronchoalveolar lavage (9), fueled the hypothesis that IgA anti-CCP would be present more frequently and detected at higher levels in smokers than in nonsmokers.

Indeed, not only were IgA anti-CCP antibodies more frequently present in smokers, but also the levels of IgA anti-CCP antibodies were higher in smokers than in nonsmokers. However, in addition to the findings regarding IgA anti-CCP antibodies, IgM anti-CCP antibodies were also more frequently detected in smokers, and the levels of all isotypes, except IgG3, as well as the number of isotypes used in the anti-CCP response were higher in smokers than in nonsmokers. These data indicate a more diverse anti-CCP response in general in patients with anti-CCP–positive RA who have been exposed to tobacco compared with patients who are nonsmokers.

Smoking not only is associated with anti-CCP–positive RA, but also has been identified as a risk factor for the development of RA among patients with anti-CCP–positive undifferentiated arthritis (UA) (10) and as a factor that influences the extent of joint damage in RA (6). The differences in isotype usage and/or the differences in levels of anti-CCP antibodies between patients with anti-CCP–positive RA who are smokers and those who are nonsmokers possibly contribute to a more severe progression of RA and a faster fulfillment of the ACR criteria within patients with UA. This is a subject of interest that should be explored further, but

was not included in the present study due to insufficient power to detect differences in disease progression.

Tobacco exposure was recently described as a contributor to the risk of anti-CCP–positive RA only among patients with SE-positive disease (8). In this study, we addressed whether the effect of smoking on the constitution of the anti-CCP response, in terms of isotype usage, was dependent on the presence of the SE as well. We observed a higher number of anti-CCP isotypes in anti-CCP–positive smokers compared with anti-CCP–positive nonsmokers, both in patients with SE-positive RA (*P* not significant, possibly as a result of a ceiling effect) and in patients with SE-negative RA ($P = 0.04$) (Figure 3). These data indicate that, at least in SE-negative RA, tobacco exposure influences the extensiveness of isotype usage in the anti-CCP response. Moreover, the results suggest that tobacco exposure is involved in the development of anti-CCP only in patients with SE-positive RA, whereas once the tolerance for citrullinated antigens is broken, the effect of tobacco exposure on the response becomes independent of T cell help via SE-bearing HLA molecules. This could, for example, be mediated by exerting a direct effect on the B cell response and/or a diversification of the underlying T cell response that now recognizes the antigen in the context of other HLA molecules.

In conclusion, patients with anti-CCP–positive RA who are current or former smokers display a more extensive anti-CCP isotype usage and a higher percentage of IgA and IgM anti-CCP antibodies than do patients with anti-CCP–positive RA who are nonsmokers. Additionally, in contrast to the influence of smoking on the presence of anti-CCP antibodies, the influence of smoking on the constitution of the anti-CCP response is not observed exclusively in patients with SE-positive RA, but also in patients with SE-negative RA, possibly reflecting the differential effects of tobacco exposure on the induction as compared with propagation of the anti-CCP response.

AUTHOR CONTRIBUTIONS

Dr. Verpoort had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Breedveld, Huizinga, Toes.

Acquisition of data. Verpoort, Papendrecht-van der Voort, Jol-van der Zijde, Drijfhout, Breedveld, de Vries.

Analysis and interpretation of data. Verpoort, Papendrecht-van der Voort, van der Helm-van Mil, Jol-van der Zijde, van Tol, Drijfhout, Huizinga, Toes.

Manuscript preparation. Verpoort, van der Helm-van Mil, Jol-van der Zijde, de Vries, Toes.

Statistical analysis. Verpoort.

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