Antibodies to *Porphyromonas gingivalis* Are Associated with Anticitrullinated Protein Antibodies in Patients with Rheumatoid Arthritis and Their Relatives

CAROL A. HITCHON, FATIHA CHANDAD, ELIZABETH D. FERUCCI, ANNEMIEK WILLEMZE, ANDREEA IOAN-FACSINAY, DIANE van der WOUDE, JANET MARKLAND, DAVID ROBINSON, BRENDA ELIAS, MARIANNA NEWKIRK, RENE M. TOES, TOM W.J. HUIZINGA, and HANI S. EL-GABALAWY

ABSTRACT. ***Objective.*** Anticitrullinated protein antibodies (ACPA) are relatively specific for rheumatoid arthritis (RA), and predate disease. The oral pathogen *Porphyromonas gingivalis* may play a role in breaking immune tolerance to citrullinated antigens. We studied a cohort of patients with RA and their relatives looking for associations between anti-*P. gingivalis* antibodies and ACPA.

Methods. Patients with RA (n = 82) and their relatives (n = 205) from a North American Native (NAN) population were studied, along with 47 NAN and 60 non-NAN controls. IgM and IgA rheumatoid factor (RF) were tested by nephelometry and ELISA. Second-generation anticyclic citrullinated peptide (anti-CCP2) isotypes and IgG anti-*P. gingivalis* lipopolysaccharides were tested by ELISA. HLA-DRB1 typing was performed by sequencing. Oral hygiene and smoking habits were assessed by questionnaires.

Results. Autoantibody frequency in patients with RA and relatives: ACPA 91% vs 19%, respectively; IgM RF 82% vs 17%; IgA RF 48% vs 22%. Anti-*P. gingivalis* levels were higher in patients with RA compared to relatives and controls ($p = 0.005$) and higher in ACPA-positive patients with RA than in ACPA-negative patients with RA ($p = 0.04$) and relatives ($p < 0.001$), but comparable in RF-positive and RF-negative patients and relatives. Poor oral hygiene and smoking were prevalent, but with no clear association with autoantibodies. Relatives with 2 shared-epitope alleles were more likely to be ACPA-positive (OR 2.5, $p = 0.02$).

Conclusion. In a genetically predisposed population of NAN patients with RA and their relatives, anti-*P. gingivalis* antibodies were associated with ACPA. These findings suggest that immune responses to *P. gingivalis* may be involved in breaking immune tolerance to citrullinated antigens. (J Rheumatol First Release May 1 2010; doi:10.3899/jrheum.091323)

Key Indexing Terms:
PORPHYROMONAS GINGIVALIS
ANTICITRULLINATED PROTEIN ANTIBODIES
RHEUMATOID ARTHRITIS
PERIODONTITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disorder centered in the synovial membrane of multiple joints. Currently, the etiology of RA remains unknown, although it has been suggested that gene-environment interactions play a substantial role in disease susceptibility[1]. Much is now known about genetic susceptibility to RA, with the HLA-DRB1 locus accounting for most of the genetic risk. Alleles of HLA-DRB1 encoding for the so-called shared-epitope (SE), a positively charged QK(R)RAA motif in the third hypervariable region of the molecule, confer RA disease susceptibility in multiple populations[2,3].

The environmental factors that potentially interact with susceptibility genes to precipitate RA onset continue to be investigated. The best documented environmental factor is tobacco smoking, which has been shown in several studies[4-9] to contribute to RA susceptibility. Importantly, smoking appears to contribute to disease susceptibility only in individuals who develop autoantibody-positive RA charac-

From the University of Manitoba, Winnipeg, Manitoba; University of Saskatchewan, Saskatoon, Saskatchewan; Laval University, Quebec City, Quebec; McGill University, Montreal, Quebec, Canada; the Alaska Native Tribal Health Consortium, Anchorage, Alaska, USA; and Leiden University Medical Center, Leiden, The Netherlands.

Supported by a research grant from the Canadian Institutes of Health Research, MOP 77000.

C.A. Hitchon, MD; D. Robinson, MD; B. Elias, PhD; H.S. El-Gabalawy, MD, University of Manitoba; F. Chandad, PhD, Laval University; E.D. Ferucci, MD, Alaska Native Tribal Health Consortium; A. Willemze, MD; A. Ioan-Facsinay, PhD; D. van der Woude, MD; R.M. Toes, PhD; T.W.J. Huizinga, MD, Leiden University Medical Center; J. Markland, MD, University of Saskatchewan; M. Newkirk, PhD, McGill University.

Address correspondence to Dr. H. El-Gabalawy, RR149, 800 Sherbrook Street, Winnipeg, Manitoba R3A 1M4, Canada.
E-mail: elgabalh@cc.umanitoba.ca

Accepted for publication February 3, 2010.

See related Editorial in this issue

terized by the presence of anticitrullinated protein antibodies (ACPA). There is a clustering of RA risk associated with smoking, carriage of SE alleles, and the presence of ACPA[6,7,10].

A number of other environmental factors have been studied for their potential role in the onset and pathogenesis of RA. Recently, several studies have reported an association between RA and periodontitis (PD)[11-13]. It has been reported that patients with longstanding active RA have a significantly increased incidence of PD compared with healthy subjects, and patients with PD have a higher prevalence of RA than patients without PD[13,14]. A recent large cross-sectional survey of the US population [National Health and Nutrition Examination Survey (NHANES) III] confirmed the documented association between RA and PD[15]. Various hypotheses have been proposed to explain the mechanisms of the potential association between RA and PD[13,16]. One of these hypotheses suggests that RA and PD may share common pathogenetic processes including common HLA-DRB1 associations, shared cellular and humoral immune reactions, and highly similar inflammatory reactions[12,15]. In addition, many therapeutic strategies for RA are also effective in PD, including the use of antiinflammatory drugs and inhibitors of cytokines and matrix metalloproteinases[17,18]. Recently it has been proposed that *Porphyromonas gingivalis*, the major etiologic agent associated with the pathogenesis of PD, may be a particularly important factor in the association between RA and PD. This association is based on the presence of the enzyme peptidyl arginine deiminase (PAD), which allows *P. gingivalis* to generate citrullinated peptides *in vivo*[13]. It is thus hypothesized that in a genetically susceptible (SE-positive) individual, such citrullinated peptides may interrupt tolerance to endogenous citrullinated antigens, resulting in the generation of an immune response to citrullinated self-antigens.

We have studied a unique cohort of North American Native (NAN) people from central Canada and have shown that the Cree/Ojibway population has some of the highest prevalence rates of RA in the world[19]. Moreover, this population, with frequent multicase families, demonstrates an early age of RA onset, high levels of ACPA and rheumatoid factor (RF), and a high population prevalence of SE alleles[20,21]. Thus, this population is an ideal model for studying disease risk in the family members of patients with RA. We have shown that almost 20% of the first-degree relatives of NAN patients with RA have detectable ACPA[21], although at this point it remains unclear whether these ACPA-positive individuals will ultimately develop RA, and over what timeframe.

Our aim was to test the hypothesis that the humoral immune response to *P. gingivalis* is associated with the presence of ACPA both in patients with well established RA and in their ACPA- positive disease-free relatives. We also attempted to relate the immune serology to RA and to oral health habits and smoking history. Our data indicate that there is indeed a clear association between the humoral immune response to *P. gingivalis* and the presence of ACPA both in patients and disease-free relatives.

MATERIALS AND METHODS

Patients, relatives, and controls. Patients with RA were recruited from a Cree and Ojibway NAN population in central Canada. Patients visiting rheumatology clinics in urban (Winnipeg, Saskatoon) and rural (Norway House, St. Theresa's Point) locations were approached to bring along unaffected relatives who were willing to participate in this study. All study subjects were over 18 years of age. The unaffected population consisted mainly of first-degree relatives (76%) and was composed of siblings and offspring, with the remainder being second-degree relatives (cousins, nieces, nephews). Eighty-five families were included in the study, comprising 82 probands and 205 relatives. All relatives had at least 1 family member with RA, but some relatives did not have their affiliated RA proband(s) included in the study, thus accounting for the larger number of families than probands. Additionally, 47 unrelated healthy controls were recruited from the same populations. Sixty white controls were randomly selected from a serum bank of healthy individuals prior to undergoing vaccination for overseas travel. The control populations were not closely matched for age and sex.

The clinical and serological characteristics of the patients with RA and

Table 1. Demographics and autoantibody status of patients with rheumatoid arthritis and relatives. For the shared-epitope, these HLA-DRB1 alleles were tested: 0101, 0102, 0103, 0301, 0401, 0402, 0403, 0404, 0407, 0408, 0410, 0701, 0801, 0802, 0811, 0901, 1101, 1103, 1301, 1302, and 1402. Shared-epitope alleles: 0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001, and 1402. Tender and swollen joint counts were based on 44-joint count; 8 relatives with joint swelling. Values are median (interquartile range) or percentage. Statistical associations tested by Mann-Whitney U or chi-square.

Characteristics	RA, N = 82	Relatives, N = 205	p
Age, yrs	52 (19)	35 (22)	< 0.001
Women, %	90	72	< 0.01
Disease duration, yrs	10 (14)	NA	—
Tender joint count	5 (11)	0 (0)	< 0.0001
Swollen joint count	4 (5)	0 (0)	< 0.0001
Erosions, %	40	NA	—
CRP, mg/l	10 (15)	4 (6)	< 0.001
ACPA any isotype, % positive	91	19	< 0.001
ACPA titer			
IgA	116 (690)	27 (14)	< 0.001
IgM	111 (229)	64 (24)	< 0.001
IgG1	412 (524)	6 (9)	< 0.001
IgG2	19 (132)	0 (2)	< 0.001
IgG3	13 (102)	0 (0)	< 0.001
IgG4	0.53 (44)	0 (0)	< 0.001
ACPA, CCP2, % positive	81	5	< 0.0001
ACPA, CCP2, titer	153 (247)	7 (8)	< 0.0001
IgM RF, % positive	82	17	< 0.001
RF titer IgM, IU	190 (554)	19 (0)	< 0.001
IgA RF, % positive	48	22	< 0.001
RF titer IgA, ELISA OD	1.2 (1.2)	0.7 (0.7)	< 0.001
Shared-epitope, %	81	73	0.2
Shared-epitope 2 copies, %	33	23	0.1

NA: not assessed; CRP: C-reactive protein; ACPA: anticitrullinated protein antibodies; CCP2: second-generation anticyclic citrullinated peptide; RF: rheumatoid factor; OD: optical density.

relatives are described in Table 1. RA was diagnosed according to the American College of Rheumatology criteria[22]. Clinical assessment of swollen, stiff, and painful joints of all patients and relatives was performed by a rheumatologist at the time of inclusion. Of the relatives, 8 presented with 1 or more swollen joints at inclusion, but did not meet criteria for RA or any other rheumatic disease, and were classified as having undifferentiated arthritis (UA). Analyses were undertaken with and without this small group of individuals with UA, and their inclusion did not affect the outcome or significance of each analysis. These individuals were analyzed as part of the unaffected relatives population. There was no difference in the demographic characteristics between the first-degree and second-degree relatives. In the patients with RA, radiographic erosions in hands and feet were assessed by review of the radiographs and/or radiograph reports, and were categorized as present or absent in each. The 47 unrelated controls had no swollen joints at inclusion and no first-degree relatives with RA.

All participants gave written consent, and the Biomedical Research Ethics Boards of the University of Manitoba and the Band Councils of each rural community approved the protocol.

ACPA antibody isotype testing. Total IgG ACPA seropositivity was assayed by ELISA using a second-generation anti-CCP kit (CCP2; Inova Diagnostics, San Diego, CA, USA). Positive tests were defined as those with values ≥ 20 units, per the manufacturer's specifications. ACPA isotypes (IgA, IgG1-4, and IgM) were measured in baseline serum samples of patients with RA, healthy relatives, and unrelated healthy controls, using CCP2 plates (Euro-Diagnostica, Arnhem, The Netherlands) and the ELISA mentioned[21]. A successive dilution of 1 reference standard, consisting of a pool of 20 ACPA-positive samples, was used in all plates. Distinct dilutions of this standard (IgA and IgM: 1:12.5; IgG1: 1:400; IgG2: 1:6.25; IgG3 and IgG4: 1:12.5) were defined as containing 1000 arbitrary units per ml (AU/ml).

Determination of cutoff values for ACPA ELISA. The level of specific antibodies in each serum sample was determined using the reference standard curve. Cutoff values for the citrullin-specific responses were calculated in each assay and were defined as 2 SD above the mean concentration of serum samples obtained from 30 healthy White controls. In case the concentrations of several control samples were below the detection limit of the ELISA, the cutoff was calculated as the lowest concentration situated on the ascending region of the standard curve. Borderline samples (with concentrations between 10 AU above and below the cutoff value) were tested at least twice for IgA ACPA and all IgG isotypes. All samples reacting with the citrullinated peptides in fine-specificity assays were retested. Only samples that were positive every time tested were considered positive.

RF measurements. IgM RF values reported throughout the study were determined by nephelometry. IgA RF and IgM RF were also measured by ELISA, using human IgG1 as the capture antigen, and F(ab')2 fragments of peroxidase-conjugated antihuman IgA or IgM, as described[23]. The cutoffs used to assign positivity were based on 60 White controls. The IgM RF values as determined by ELISA were comparable to those obtained by nephelometry.

Humoral immune response against P. gingivalis antigens. Lipopolysaccharides (LPS) of *P. gingivalis* were isolated by the method described by Darveau and Hancock[24]. The amount of contaminating proteins was evaluated using a protein assay kit (Biorad Laboratories, Mississauga, ON, Canada) and was < 0.001%.

Antibodies to P. gingivalis LPS and ELISA index calculations. IgG antibodies specific to LPS of *P. gingivalis* were measured using ELISA. The wells of 96-well flat-bottom microtiter plates were coated in triplicate with LPS of *P. gingivalis*. After washing and blocking the plates, serum samples were added to individual wells and specific human IgG antibodies were detected with an alkaline phosphatase-conjugated antihuman immunoglobulin. The absorbance was read at 405 nm using an ELISA plate reader. Each assay included a conjugate control, a substrate control, a conjugate-substrate reactivity control, and a serially diluted reference serum control. The results were expressed as an ELISA Index (EI), which was the mean OD405 of a given serum divided by the mean OD405 of the calibrator (reference serum). Positive calibrator and negative assay controls were included in each run to control for intraassay and interassay variation. Values were reported as median (interquartile range; IQR).

Smoking and oral health questionnaires. Subjects answered questionnaires regarding smoking and oral hygiene habits. Smoking was assessed by the duration and the number of smoked cigarettes [i.e., 1 cigarette/day for 3 or more months, at least 5 cigarettes in the past 6 months (current smoker); 1–5 cigarettes/month, 1–5 cigarettes/week, fewer than 10 cigarettes/day, half to 1 pack/day, or more than 1 pack/day). Oral hygiene habits were assessed by asking participants how many times a week they brushed and flossed their teeth, on a scale of never, 1–6 times, 7–14 times, or 15+ times. They also were asked how often they see a dentist, on a scale of never, 1–2 times/year, or 2+ times/year.

Statistical analysis. Statistical analyses were performed using the SPSS (version 14.0) software. Data distribution was tested for normality, and non-normally distributed data reported as median and IQR. Mann-Whitney U or Kruskal-Wallis tests were used to compare age, joint counts, C-reactive protein, and levels of *P. gingivalis*, ACPA isotypes, and RF. Differences in the distribution of the ACPA isotypes between healthy relatives and patients with RA and associations with dental habits and smoking were calculated using the chi-squared test. The chi-squared test was also used for calculating OR with 95% CI, and p values for the association of SE with ACPA. When a cell contained < 5 individuals, Fisher's exact 2-tailed p value was calculated. A p value < 0.05 and a 95% CI that excluded the value of 1 were considered significant.

RESULTS

The clinical characteristics of the study subjects are shown in Table 1. The relatives were younger than patients, and less likely to be women. As reported[21], 91% of patients and 19% of relatives had ACPA of at least 1 isotype, and patients had a greater number of ACPA isotypes and higher ACPA levels. Similar findings were seen using a CCP2 kit that measures only total IgG ACPA (RA 81% vs relatives 5% positive). There were no significant differences between patients and relatives in the presence of SE alleles (81% vs 73%) or in the presence of 2 copies of SE alleles (33% vs 23%), although both tended to be higher in patients. Relatives with 2 SE alleles were more likely to be ACPA-positive (OR 2.5, p = 0.02). HLA-DRB1*0404 and *1402 are the most prevalent SE alleles in this population[20].

IgG antibody responses to *P. gingivalis* LPS were measured in the sera from patients with RA (n = 82), relatives (n = 205), unrelated NAN controls from the same population (n = 47), and non-NAN controls from a serum bank (n = 60). Five patients with RA and 9 relatives had samples that could not be analyzed for anti-*P. gingivalis* LPS responses, but these individuals did not differ significantly from the remainder of the cohort. As shown in Figure 1, anti-*P. gingivalis* levels were higher in the patients with RA compared to the other groups combined [median (IQR) 42 (30) vs 33 (19) AU; p = 0.001], but there were no differences between the relatives and the 2 control groups. Patients with RA with and without erosions did not differ in anti-*P. gingivalis* levels (data not shown).

We next looked specifically at the association between anti-*P. gingivalis* responses and ACPA serology in patients with RA and relatives. Levels of ACPA isotypes (IgG1-4,

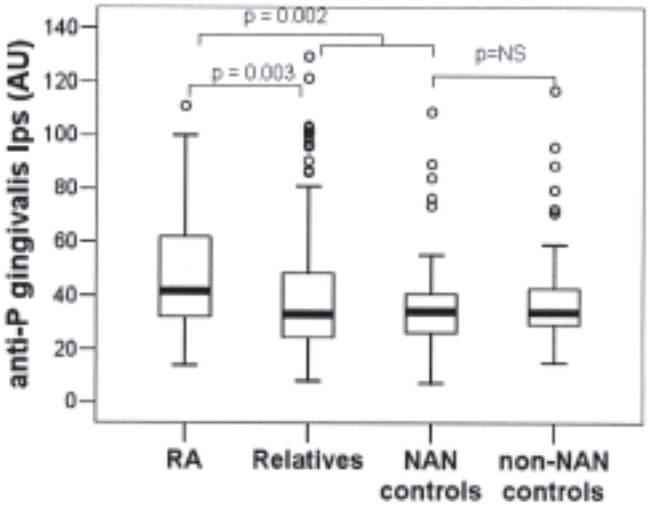

Figure 1. Levels of anti-*P. gingivalis* ELISA index in patients with RA, relatives, First Nations (NAN) controls, and non-NAN controls. Numbers represent arbitrary units derived from ELISA optical densities.

IgA, IgM) were analyzed by ELISA as described. Individuals were considered ACPA-positive if their ELISA levels were above the cutoff threshold established for any of the ACPA isotypes. The data in Figure 2A indicate that anti-*P. gingivalis* responses were significantly higher in ACPA-positive than in ACPA-negative individuals. In the unaffected relatives group, ACPA-positive individuals had higher anti-*P. gingivalis* LPS levels [median (IQR) 44 (21) vs 32 (22) AU; $p < 0.0001$] compared to ACPA-negative relatives. In the group of patients with RA, ACPA-positive individuals had higher anti-*P gingivalis* levels compared to ACPA-negative patients with RA [median (IQR) 43 (29) vs 25 (28) AU; $p = 0.04$] even though only 6/82 patients (7%) were ACPA-negative. There was no difference in anti-*P. gingivalis* levels between ACPA-positive relatives and ACPA-positive patients with RA. Moreover, of the ACPA-positive relatives and patients, there were no significant differences in the anti-*P. gingivalis* levels for each individual isotype (data not shown). These data indicate that there is an association between humoral immune responses to *P. gingivalis* and ACPA positivity, and that this association is independent of having RA itself. In contrast, there was no association between anti-*P. gingivalis* antibody levels and RF, as shown in Figure 2B. Further, as shown in Table 2, after stratifying for ACPA in the IgM RF-positive or IgA RF-negative individuals, the association of *P. gingivalis* levels with ACPA remained in the relatives, but not in the patients with RA. In contrast, in ACPA-positive individuals there were no associations of IgM or IgA RF with anti-*P. gingivalis* levels. This supports a primary association between ACPA positivity and *P. gingivalis* levels.

We next examined whether poor oral health habits were associated with immune responses to *P. gingivalis*, with the presence of ACPA, or with RA. A subset of subjects (56 patients; 141 relatives) answered a detailed questionnaire regarding oral hygiene habits. There were no significant clinical, serological, or anti-*P. gingivalis* antibody level differences between subjects who did or did not answer the oral hygiene questions. As shown in Table 3, patients with RA were more likely to have dentures (37% vs 15%; $p < 0.001$) compared to relatives, and visited the dentist less often. Both groups had similar oral hygiene habits, with 44% of patients and 55% of relatives brushing at least once daily and > 60% flossing. There were no associations between oral hygiene habits and ACPA positivity apart from an increased tendency to have dentures.

Since smoking has been associated in studies with both ACPA[5,7] and PD[25,26], we investigated whether there was any association between smoking habits and the humoral

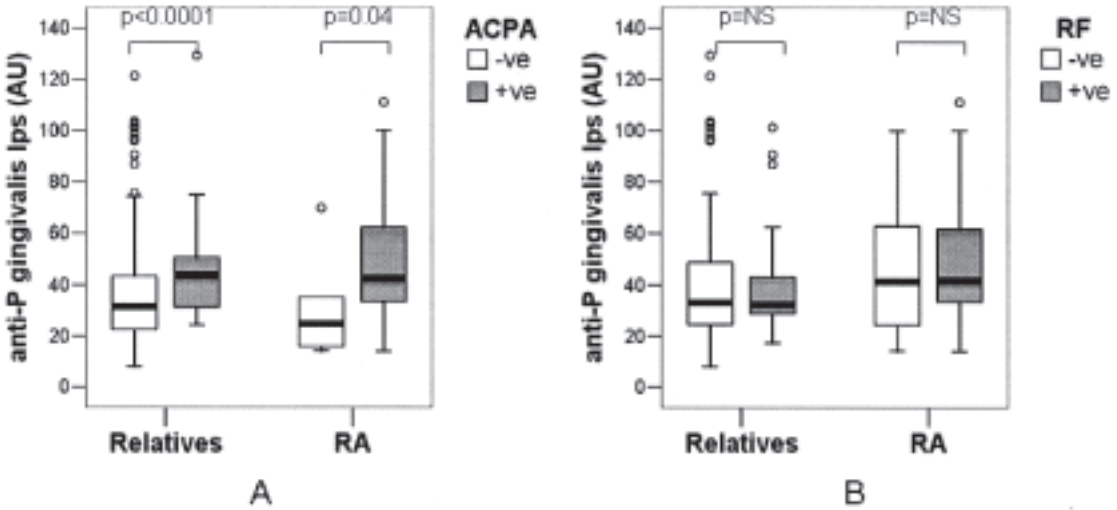

Figure 2. Levels of anti-*P. gingivalis* in relatives and patients with RA according to the presence of (A) anticitrullinated protein antibodies (ACPA) or (B) rheumatoid factor (RF). Numbers represent arbitrary units derived from ELISA optical densities.

Table 2. Association of anti-*P. gingivalis* lipopolysaccharide levels and IgM RF, IgA RF, and ACPA. Values are median (interquartile range) of anti-*P. gingivalis* levels in patients with RA or their relatives with IgM rheumatoid factor (IgM RF), IgA RF, and/or anticitrullinated protein antibodies (ACPA); p values were calculated using Mann-Whitney U tests.

	RA	Relatives
IgM RF-negative		
ACPA-negative	30 (35) (n = 5)	32 (22) (n = 130)
ACPA-positive	48 (38) (n = 9)	46 (23) (n = 30)
	p = 0.21	p = 0.001
IgM RF-positive		
ACPA-negative	NA (n = 1)	31 (20) (n = 25)
ACPA-positive	42 (29) (n = 62)	41 (14) (n = 10)
	p = 0.095	p = 0.14
IgA RF-negative		
ACPA-negative	20 (17) (n = 5)	31 (22) (n = 118)
ACPA-positive	40 (22) (n = 35)	42 (21) (n = 24)
	p = 0.008	p = 0.007
IgA RF-positive		
ACPA-negative	NA (n = 1)	37 (20) (n = 28)
ACPA-positive	44 (34) (n = 34)	47 (20) (n = 12)
	p = 0.28	p = 0.25
ACPA-negative		
IgM RF-negative	30 (35) (n = 5)	32 (22) (n = 124)
IgM RF-positive	NA (n = 1)	32 (20) (n = 22)
	p = 0.67	p = 0.75
ACPA-positive		
IgM RF-negative	48 (38) (n = 9)	46 (21) (n = 28)
IgM RF-positive	42 (30) (n = 60)	41 (13) (n = 8)
	p = 0.68	p = 0.25
ACPA-negative		
IgA RF-negative	20 (17) (n = 5)	31 (22) (n = 118)
IgA RF-positive	NA (n = 1)	37 (20) (n = 28)
	p = 0.33	p = 0.14
ACPA-positive		
IgA RF-negative	40 (22) (n = 35)	42 (21) (n = 24)
IgA RF-positive	44 (34) (n = 34)	47 (20) (n = 12)
	p = 0.23	p = 0.75

NA: not available because only 1 subject.

immune response to *P. gingivalis* and to ACPA positivity. The data shown in Table 3 indicate that 75% of patients with RA and 74% of relatives had smoked at least 1 cigarette/day for at least 3 months, while 22% of patients with RA and 23% of relatives smoked more than a half pack/day. On the basis of these similar high smoking rates in both patients with RA and relatives, no clear association could be established between smoking-related measurements and the immune response to *P. gingivalis* or ACPA positivity. Moreover, no association was seen between smoking, SE (single or 2 copies), and ACPA of any isotype in patients with RA or relatives. There was no association between anti-*P. gingivalis* levels and SE.

DISCUSSION

There has been a well documented association between RA and PD since the 1950s, although the biological basis for this association has not been clearly elucidated. This epidemiological association was confirmed in the NHANES III study, which suggested an OR 1.82, 95% CI 1.04–3.20[15]. It has been hypothesized that this association may be based on the capacity of *P. gingivalis*, the major etiological agent of periodontitis, to express a PAD, an enzyme responsible for posttranslational citrullination of arginine residues[13]. This enzymatic activity potentially exposes affected individuals to citrullinated antigens, and in the context of the appropriate immunogenetic background, would predispose to the development of ACPA. ACPA are known to be relatively specific for RA, to precede disease onset, and to potentially be involved in the pathogenesis of RA synovitis[27,28].

We investigated whether there was an association between immune responses to the periodontal pathogen *P. gingivalis* and the presence of RA and/or ACPA. Sera from a cohort of NAN patients with RA and their unaffected relatives who are at risk for disease development[21] were analyzed for ACPA, RF, and specific IgG antibodies to *P. gingivalis* LPS. We demonstrate that serum levels of the anti-*P. gingivalis* LPS were higher in patients with RA compared to their relatives, and to 2 distinct control groups, 1 of which was from the same population. Importantly, the data reveal that the immune responses to *P. gingivalis* LPS were significantly higher in ACPA-positive than in ACPA-negative individuals, irrespective of whether they had RA.

Mikuls, *et al* recently compared the levels of anti-*P. gingivalis* antibodies in patients with RA, PD, and healthy controls[29]. These data showed that the levels of anti-*P. gingivalis* antibodies were highest in PD, lowest in controls, and intermediate in RA. This study also showed an association between the levels of anti-*P. gingivalis* antibodies and levels of IgM and IgG2 ACPA, but not RF, in the patients with RA. Our study is consistent with these data. It should be pointed out that we tested the antibody response to a more restricted *P. gingivalis* antigen, LPS, while the Mikuls study evaluated antibody responses to a broader antigenic spectrum using lysates of whole organisms.

We present data from a unique cohort of the disease-free relatives of patients with RA who have a high prevalence of ACPA. This allowed us to address a key question: whether antibody responses to *P. gingivalis* are associated with ACPA outside the context of RA. The data clearly indicate that this is the case. Moreover, this association was specific for ACPA as there was no association with RF. The lack of association between antibody responses to *P. gingivalis* and RF is also reported in the Mikuls study[29]. The findings are consistent with the hypothesis that immune responses to *P. gingivalis* are in some way involved with the breaking of immune tolerance to citrullinated antigens, as indicated by the presence of ACPA. Since *P. gingivalis* expresses PAD and can potentially citrullinate peptides *in vivo*, host immune responses to such neoantigens may trigger autoimmunity to endogenous citrullinated antigens through mechanisms such as molecular mimicry and epitope-spreading[13].

Table 3. Self-reported dental hygiene and smoking habits and their associations with ACPA and levels of anti-*P. gingivalis* lipopolysaccharide. Values are number of subjects with RA or their relatives reporting habit. Anti-*P. gingivalis* levels reported as median (interquartile range) of arbitrary units in patients with RA and relatives.

Oral Hygiene and Smoking Habit	RA vs Relatives			RA and Relatives				
	RA	Relatives	p	ACPA Negative	ACPA Positive	p	Anti-*P. gingivalis* Levels Level	p
Dental examination								
Never	9	10	0.02	8	11	0.05	26 (45)	0.8
> 1/year	34	117		99	52		32 (20)	
Floss								
No	14	39	0.60	34	19	0.72	31 (15)	0.36
Yes	24	82		71	35		32 (36)	
Brush								
< 1/day	23	57	0.21	49	31	0.35	32 (34)	0.84
= 1/day	18	70		60	28		30 (20)	
Dentures								
No	32	110	0.001	94	48	0.007	32 (23)	0.47
Yes	19	19		16	22		29 (24)	
Ever smoker								
Nonsmoker	19	50	0.87	39	30	0.49	36 (NA)	0.09
= 1/cig/day for 3 months	58	143		123	78		31 (22)	
Amount smoked								
1-5/month	1	10	0.43	8	3	0.69	29 (26)	0.99
1-5/week	9	21		18	12		34 (26)	
< half pack per day	24	70		60	34		33 (22)	
half-1 pack per day	10	29		25	14		31 (19)	
> 1 pack per day	0	3		3	0		39 (NA)	

NA: not available because only 3 subjects.

It is currently unknown which peptides or proteins may constitute the most important *in vivo* antigen for ACPA, but several citrullinated proteins have been reported as possible targets, including citrullinated fibrinogen, vimentin, type II collagen, and α-enolase[30-33]. Citrullinated α-enolase peptide 1 (CEP-1), the immunodominant peptide of human α-enolase, has 82% homology with enolase from *P. gingivalis,* and anti-CEP1 antibodies have been described to be cross-reactive with the bacterial enzyme[34]. On the basis of these data, it was concluded that bacterial infection with *P. gingivalis* may play a role in priming the ACPA response. Although antibody responses to CEP-1 were not measured as part of our study, we have used a different α-enolase peptide in our fine specificity studies[21]. This peptide (called C6) was recognized by 14% of NAN patients with RA and none of the relatives, thus precluding a meaningful analysis in the context of our study. Large-scale studies and longitudinal followup of this cohort, to determine which of the ACPA-positive relatives may develop RA, will be required to more clearly elucidate the possible associations between ACPA, antienolase reactivity, and *P. gingivalis*.

We attempted to determine whether oral hygiene and smoking were associated with RA, ACPA, and with immune responses to *P. gingivalis*. It is known that smoking and poor oral health habits both increase the risk of PD. Smoking is a risk factor for PD possibly through the effects of nicotine on inflammatory cytokine profiles[35,36] and matrix metalloproteinase activity[37,38] or potentially even through direct effects on *P. gingivalis* gene expression[39]. We did find a high prevalence of both smoking and poor oral health habits in the study population based on self-report questionnaire data; however, we could not define a clear association between these factors and the presence of either RA or ACPA. Studies of NAN populations from a similar geographical area have shown a high prevalence of PD that is associated with poor dental hygiene habits[40]. Further studies, including formal dental assessments and sampling, are under way to confirm the presence and extent of PD in this population.

The reported association between immune responses to the oral pathogen *P. gingivalis* and the presence of ACPA in a population with a high background prevalence of RA-predisposing HLA-DRB1 alleles is consistent with a gene-environment interaction that may result in breaking self-tolerance to citrullinated antigens and/or amplification of these autoimmune responses, and ultimately leading to the development of RA. It should be added that the sample size tested in this study precluded an analysis of such as gene-environment interaction. Moreover, the demonstrated association between *P. gingivalis* and ACPA may reflect a broader association with periodontitis, which was not addressed by systematic oral examinations. While further studies are needed to support this association and to assess longitudinal

outcomes in high-risk individuals, we suggest that interventions directed at modulating these environmental risk factors, such as improved oral health and smoking cessation, may play an important role in reducing or delaying the onset of future RA.

ACKNOWLEDGMENT

We thank the First Nations communities, their chiefs, and band councils for support during this study.

REFERENCES

1. Klareskog L, Padyukov L, Lorentzen J, Alfredsson L. Mechanisms of disease: Genetic susceptibility and environmental triggers in the development of rheumatoid arthritis. Nat Clin Pract Rheumatol 2006;2:425-33.
2. Kochi Y, Suzuki A, Yamada R, Yamamoto K. Genetics of rheumatoid arthritis: underlying evidence of ethnic differences. J Autoimmun 2009;32:158-62.
3. Oen K, Robinson DB, Nickerson P, Katz SJ, Cheang M, Peschken CA, et al. Familial seropositive rheumatoid arthritis in North American Native families: effects of shared epitope and cytokine genotypes. J Rheumatol 2005;32:983-91.
4. Heliovaara M, Aho K, Aromaa A, Knekt P, Reunanen A. Smoking and risk of rheumatoid arthritis. J Rheumatol 1993;20:1830-5.
5. Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum 2006;54:38-46.
6. Lundstrom E, Kallberg H, Alfredsson L, Klareskog L, Padyukov L. Gene-environment interaction between the DRB1 shared epitope and smoking in the risk of anti-citrullinated protein antibody-positive rheumatoid arthritis: all alleles are important. Arthritis Rheum 2009;60:1597-603.
7. Pedersen M, Jacobsen S, Garred P, Madsen HO, Klarlund M, Svejgaard A, et al. Strong combined gene-environment effects in anti-cyclic citrullinated peptide-positive rheumatoid arthritis: a nationwide case-control study in Denmark. Arthritis Rheum 2007;56:1446-53.
8. Silman AJ, Newman J, MacGregor AJ. Cigarette smoking increases the risk of rheumatoid arthritis. Results from a nationwide study of disease-discordant twins. Arthritis Rheum 1996;39:732-5.
9. Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, et al. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. Ann Rheum Dis 2003;62:835-41.
10. van der Helm-van Mil AH, Verpoort KN, le Cessie S, Huizinga TW, de Vries RR, Toes RE. The HLA-DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide. Arthritis Rheum 2007;56:425-32.
11. Bartold PM, Marshall RI, Haynes DR. Periodontitis and rheumatoid arthritis: a review. J Periodontol 2005;76:2066-74.
12. Marotte H, Farge P, Gaudin P, Alexandre C, Mougin B, Miossec P. The association between periodontal disease and joint destruction in rheumatoid arthritis extends the link between the HLA-DR shared epitope and severity of bone destruction. Ann Rheum Dis 2006;65:905-9.
13. Rosenstein ED, Greenwald RA, Kushner LJ, Weissmann G. Hypothesis: the humoral immune response to oral bacteria provides a stimulus for the development of rheumatoid arthritis. Inflammation 2004;28:311-8.
14. Mercado FB, Marshall RI, Klestov AC, Bartold PM. Relationship between rheumatoid arthritis and periodontitis. J Periodontol 2001;72:779-87.
15. de Pablo P, Dietrich T, McAlindon TE. Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. J Rheumatol 2008;35:70-6.
16. Ribeiro J, Leao A, Novaes AB. Periodontal infection as a possible severity factor for rheumatoid arthritis. J Clin Periodontol 2005;32:412-6.
17. Greenwald RA, Kirkwood K. Adult periodontitis as a model for rheumatoid arthritis (with emphasis on treatment strategies). J Rheumatol 1999;26:1650-3.
18. Rosenstein ED, Kushner LJ, Kramer N, Kazandjian G. Pilot study of dietary fatty acid supplementation in the treatment of adult periodontitis. Prostaglandins Leukot Essent Fatty Acids 2003;68:213-8.
19. Peschken CA, Esdaile JM. Rheumatic diseases in North America's indigenous peoples. Semin Arthritis Rheum 1999;28:368-91.
20. El-Gabalawy HS, Robinson DB, Hart D, Elias B, Markland J, Peschken CA, et al. Immunogenetic risks of anti-cyclical citrullinated peptide antibodies in a North American Native population with rheumatoid arthritis and their first-degree relatives. J Rheumatol 2009;36:1130-5.
21. Ioan-Facsinay A, Willemze A, Robinson DB, Peschken CA, Markland J, van der Woude D, et al. Marked differences in fine specificity and isotype usage of the anti-citrullinated protein antibody in health and disease. Arthritis Rheum 2008;58:3000-8.
22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
23. Newkirk MM, Goldbach-Mansky R, Senior BW, Klippel J, Schumacher HR Jr, El-Gabalawy HS. Elevated levels of IgM and IgA antibodies to Proteus mirabilis and IgM antibodies to Escherichia coli are associated with early rheumatoid factor (RF)-positive rheumatoid arthritis. Rheumatology 2005;44:1433-41.
24. Darveau RP, Hancock RE. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough Pseudomonas aeruginosa and Salmonella typhimurium strains. J Bacteriol 1983;155:831-8.
25. Do LG, Slade GD, Roberts-Thomson KF, Sanders AE. Smoking-attributable periodontal disease in the Australian adult population. J Clin Periodontol 2008;35:398-404.
26. Preshaw PM, Seymour RA, Heasman PA. Current concepts in periodontal pathogenesis. Dent Update 2004;31:570-8.
27. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum 2004;50:380-6.
28. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003;48:2741-9.
29. Mikuls TR, Payne JB, Reinhardt RA, Thiele GM, Maziarz E, Cannella AC, et al. Antibody responses to Porphyromonas gingivalis (P. gingivalis) in subjects with rheumatoid arthritis and periodontitis. Int Immunopharmacol 2009;9:38-42.
30. Burkhardt H, Sehnert B, Bockermann R, Engstrom A, Kalden JR, Holmdahl R. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. Eur J Immunol 2005;35:1643-52.
31. Kinloch A, Tatzer V, Wait R, Peston D, Lundberg K, Donatien P, et al. Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. Arthritis Res Ther

2005;7:R1421-9.
32. Masson-Bessiere C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, et al. The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. J Immunol 2001;166:4177-84.
33. Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, et al. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. Arthritis Res Ther 2004;6:R142-50.
34. Lundberg K, Kinloch A, Fisher BA, Wegner N, Wait R, Charles P, et al. Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase. Arthritis Rheum 2008;58:3009-19.
35. de Heens GL, Kikkert R, Aarden LA, van der Velden U, Loos BG. Effects of smoking on the ex vivo cytokine production in periodontitis. J Periodontal Res 2009;44:28-34.
36. de Heens GL, van der Velden U, Loos BG. Cigarette smoking enhances T cell activation and a Th2 immune response; an aspect of the pathophysiology in periodontal disease. Cytokine 2009;47:157-61.
37. Zhang W, Song F, Windsor LJ. Cigarette smoke condensate affects the collagen-degrading ability of human gingival fibroblasts. J Periodontal Res 2009;44:704-13.
38. Ho YC, Yang SF, Huang FM, Chang YC. Up-regulation of osteolytic mediators in human osteosarcoma cells stimulated with nicotine. J Periodontal Res 2009;44:760-6.
39. Bagaitkar J, Williams LR, Renaud DE, Bemakanakere MR, Martin M, Scott DA, et al. Tobacco-induced alterations to Porphyromonas gingivalis-host interactions. Environ Microbiol 2009;11:1242-53.
40. Brothwell D, Ghiabi E. Periodontal health status of the Sandy Bay First Nation in Manitoba, Canada. Int J Circumpolar Health 2009;68:23-33.