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Malondialdehyde-Acetaldehyde Adducts and Anti–Malondialdehyde-Acetaldehyde Antibodies in Rheumatoid Arthritis

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Objective. **Malondialdehyde-acetaldehyde (MAA) adducts are a product of oxidative stress associated with tolerance loss in several disease states. This study was undertaken to investigate the presence of MAA adducts and circulating anti-MAA antibodies in patients with rheumatoid arthritis (RA).**

Methods. **Synovial tissue from patients with RA and patients with osteoarthritis (OA) were examined for the presence of MAA-modified and citrullinated proteins. Anti-MAA antibody isotypes were measured in RA patients (n = 1,720) and healthy controls (n = 80) by enzyme-linked immunosorbent assay. Antigen-specific anti–citrullinated protein antibodies (ACPAs) were measured in RA patients using a multiplex antigen array. Anti-MAA isotype concentrations were compared in a subset of RA patients (n = 80) and matched healthy controls (n = 80). Associations of anti-MAA antibody isotypes with disease characteristics, including ACPA positivity, were examined in all RA patients.**

Results. **Expression of MAA adducts was increased in RA synovial tissue compared to OA synovial tissue, and colocalization with citrullinated proteins was found. Increased levels of anti-MAA antibody isotypes were observed in RA patients compared to controls ($P < 0.001$). Among RA patients, anti-MAA antibody isotypes were associated with seropositivity for ACPAs and rheumatoid factor ($P < 0.001$) in addition to select measures of disease activity. Higher anti-MAA antibody concentrations were associated with a greater number of positive antigen-specific ACPA analytes (expressed at high titer) ($P < 0.001$) and a higher ACPA score ($P < 0.001$), independent of other covariates.**

Conclusion. **MAA adduct formation is increased in RA and appears to result in robust antibody responses that are strongly associated with ACPAs. These results support speculation that MAA formation may be a cofactor that drives tolerance loss, resulting in the autoimmune responses characteristic of RA.**

Lipid peroxidation leading to the formation of protein adducts promotes proinflammatory responses that characterize a variety of chronic health conditions and related environmental exposures, including cardiovascular disease, alcoholic liver disease, and cigarette smoking (1–4). Malondialdehyde (MDA) is one such ubiquitous product implicated in disease pathogenesis. When cells are exposed to reactive oxygen species, lipid peroxidation occurs, causing cell walls to rupture and membrane lipids to oxidize into MDA (5). Moreover, MDA can spontaneously break down and form acetaldehyde (AA) (6). Importantly, both MDA and AA are

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highly reactive aldehydes, and together have been demonstrated to modify proteins to produce an MDA-AA protein adduct, termed malondialdehyde-acetaldehyde (MAA). In contrast to MAA adducts, which are highly immunogenic and characterized by a stable ring structure (7,8), MDA and AA by themselves are relatively unstable and either rapidly dissociate or form other metabolic products (9,10).

Although there have been no studies examining the role of MAA in rheumatoid arthritis (RA), several investigations have shown that the levels of oxidative stress, including expression of MDA, are increased in patients with RA (11–14). Compared to healthy controls and patients with osteoarthritis (OA), patients with RA have higher levels of circulating MDA (15). Moreover, compared to patients with seronegative RA, both circulating and synovial levels of MDA are increased in those with seropositive disease (16).

Although little is known about MAA adducts in RA, they are overexpressed in a variety of diseased tissues, act as immunodominant epitopes, and promote robust inflammatory responses (17). Previous studies have demonstrated that MAA-modified proteins elicit isotype-specific antibody responses and induce the expression of proinflammatory cytokines, including tumor necrosis factor α (TNFα), intracellular adhesion molecule 1, and vascular cell adhesion molecule (2,18). Animal studies have demonstrated that immunization with MAA-modified bovine serum albumin in the absence of adjuvant induces robust antibody, T helper cell, and cytotoxic T cell responses to MAA epitopes—all furthering the belief that MAA has a causal relationship with immunologic responses (7,19,20). Additional studies have shown that MAA adducts are immunogenic even in the absence of adjuvant (7), and their presence results in adaptive immune responses to the carrier protein itself, thereby bypassing tolerance (21).

In this study, we examined whether MAA was present in RA synovium, and if so, whether MAA adducts potentially mediate the initiation and/or progression of related immune responses. Furthermore, given the proposed role of MAA modification in tolerance loss, we examined whether anti-MAA antibody responses could serve as a marker of increased disease-specific autoantibody responses in patients with RA.

PATIENTS AND METHODS

Study subjects. To determine whether MAA is present in RA synovium, we examined synovial tissue samples from 3 patients with RA who were positive for anti–cyclic citrullinated peptide (anti-CCP) antibodies and from 3 patients with OA obtained at the time of total joint arthroplasty (all tissue samples obtained from the laboratory of Dr. Robinson at Stanford University). Serologic analyses were performed using data and serum samples from patients with RA in the Veterans Affairs Rheumatoid Arthritis (VARA) registry (22). The registry has received institutional review board approval, and all patients provided written informed consent prior to enrollment. This study was also approved by the VARA Scientific and Ethics Advisory Committee.

All participants with RA satisfied the American College of Rheumatology 1987 classification criteria for RA (23). To examine whether circulating concentrations of anti-MAA antibodies were higher in the context of RA, we also performed assays on banked samples from a convenience cohort of 80 volunteer controls. The control subjects were individuals who reported no systemic inflammatory diseases, including RA, and all but 4 of the control subjects reported good-to-excellent overall health status (24).

Chemicals, proteins, and antibodies. Aqueous human albumin was purchased from Talecris Biotherapeutics. AA was obtained from Aldrich Chemistry. MDA was obtained in sodium salt form by treatment of tetramethoxypropane (Aldrich Chemistry) with NaOH, according to the method described by Kikugawa et al (25). Phytic acid (PA) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma. For detection of citrullinated proteins, the anticitrulline monoclonal antibody F95 was used. This anticitrulline antibody is a mouse IgM antibody that has been shown to react with all citrullinated proteins (26). The anti-MAA polyclonal antibody used was developed in our laboratories. This antibody is a rabbit IgG antibody that has been shown to react with the 1,4-dihydropyridine adduct formed when MDA and AA are present (27).

Preparation of modified proteins. In order to evaluate the specificity of antibody responses to citrullinated or MAA-modified structures, aqueous human albumin was used, in the following forms: unmodified Alb = no modification; Alb-Cit = citrullinated albumin; Alb-MAA = MAA-modified albumin; Alb-Cit-MAA = albumin citrullinated and then MAA modified. Albumin was citrullinated using rabbit skeletal muscle peptidylarginine deiminase (Sigma), as previously described (28). Verification of citrullination, resulting in the formation of Alb-Cit and Alb-Cit-MAA, was determined by enzyme-linked immunosorbent assay (ELISA) using the anticitrulline monoclonal antibody (26). Albumin was adjusted to 2 mg and modified by reacting with 2 m*M* MDA and 1.0 m*M* AA in 0.1*M* phosphate buffer, pH 7.2, containing 2 m*M* DTPA and 2 m*M* PA, at 37°C for 3 days, followed by dialysis against 3 changes of 0.1*M* sodium phosphate buffer for 24 hours at 4°C (8). Alb-MAA and Alb-Cit-MAA were assayed for modification by the amount of fluorescent MAA adduct present (excitation wavelength 398 nm and emission wavelength 460 nm) using a Turner Biosystems LS-5B spectrofluorimeter.

Immunohistochemical staining of synovial tissue. Synovial tissue was collected from 3 RA patients and 3 OA patients, as described above, and placed in OCT compound. Frozen sections were prepared and blocked with normal goat serum. The sections were stained for the presence of MAA-modified proteins using the MAA-specific rabbit polyclonal antibody. In addition, the sections were stained for citrulli-

nated proteins using the citrulline-specific mouse IgM monoclonal antibody, and stained for leukocytes using a rat IgG2b anti-CD45 monoclonal antibody (AbD Serotec). The specific reactivity of the anti-MAA and anticitrulline antibodies in binding MAA-modified protein or citrullinated protein, respectively, has been well documented (26,27).

These antibodies were chosen to ensure that the labeled secondary antibodies specifically reacted with the primary antibodies. Briefly, all antibodies were diluted 1:100 and incubated with the synovial tissue sections overnight at 4°C. Sections were washed and incubated with the following secondary antibodies (all diluted 1:1,000; Jackson ImmunoResearch): tetramethylrhodamine isothiocyanate–conjugated AffiniPure goat anti-mouse IgM (5μ Fc fragment–specific) to detect the anticitrulline antibody, Dylight 405–conjugated AffiniPure goat anti-rabbit IgG (Fc-specific) to detect the anti-MAA antibody, and Dylight 649–conjugated AffiniPure mouse anti-rat IgG (Fc-specific) to detect the anti-CD45 antibody. After incubation for 45 minutes at room temperature, the slides were washed, mounted, and imaged using a Zeiss 510 Meta confocal laser scanning microscope. All images were analyzed using Zen 2012 software (Zeiss). Image quantification was done using ImageJ software (National Institutes of Health). Results are expressed as the mean $\pm$ SD pixel density.

Characteristics of patients with RA. The VARA registry includes standardized clinical data obtained from patients during routine care. Enrollment variables include RA classification criteria, comorbidities, cigarette smoking status (never, former, or current), sociodemographic features (education, race/ethnicity, age, sex), body mass index, date of RA diagnosis, and medication use (including prednisone, methotrexate, and anti-TNFα therapy). Anti–citrullinated protein antibodies (ACPAs) were measured in banked serum samples at baseline using a second-generation anti-CCP antibody ELISA (cutoff for positivity $\geq$5 units/ml) (Diastat; Axis-Shield Diagnostics). Rheumatoid factor (RF) positivity (cutoff for positivity $\geq$15 IU/ml) and high-sensitivity C-reactive protein levels (in mg/liter) were determined by nephelometry (Siemens Healthcare Diagnostics). Additional measures collected at enrollment included the tender and swollen joint counts (range for each 0–28) and the erythrocyte sedimentation rate (ESR; in mm/hour). The 4-variable Disease Activity Score in 28 joints (DAS28) (29) was used to assess levels of disease activity.

Determination of circulating antibodies to MAA. Serum samples from all RA patients were screened for the presence of the IgM, IgG, and IgA isotypes of anti-MAA antibodies, as previously described (17). Briefly, aqueous human albumin was modified with MDA and AA (ratio of 2:1) (27). ELISA plates were coated with 2 μg/well of either MAA-Alb or unmodified Alb. Additional wells were coated with known concentrations of human IgM, IgG, or IgA isotype standards (Sigma), from which the relative antibody concentrations were extrapolated. Plates were incubated overnight at 4°C and then washed, blocked with 2% bovine serum albumin, and incubated with patient serum at a 1:1,000 dilution. Following incubation at 37°C for 1 hour, a secondary horseradish peroxidase (HRP)–conjugated goat anti-human antibody specific for IgM (5μ Fc fragment–specific), IgG (Fcγ-specific), or IgA (α-chain–specific) (Jackson ImmunoResearch) was added. Plates were developed using tetramethylbenzidine (TMB) substrate, and the absorbance at 450 nm was determined using an MRX II microplate reader (Dyantech). Data are presented as arbitrary units (AUs) of the specific isotype detected in the assay, since this reflects the amount of antibody present in a sample relative to a standard curve.

Determination of cross-reactivity by ELISA. Cross-reactivity between the anti-MAA and anti-CCP assays was assessed. For these experiments, 96-well Immulon IV microtiter plates (Nunc; Fisher Scientific) were coated with 2 μg/well unmodified Alb, Alb-Cit, Alb-MAA, or Alb-Cit-MAA in bicarbonate buffer (pH 9.6), as previously described (7). The rabbit anti-MAA antibody and the mouse anticitrulline antibody were each diluted 1:1,000, yielding a reactivity to their matching antigen of 1.5 optical density (OD). This reactivity represents the dilution that results in one-half maximum binding in our ELISAs. Similar dilutions of 5 patient samples from each of the following 3 groups were also prepared; anti-MAA–positive/anticitrulline-negative, anti-MAA–negative/anticitrulline-positive, or anti-MAA–positive/anticitrulline-positive. In a separate incubation plate, these samples were incubated at 4°C overnight with 2-fold dilutions of phosphate buffered saline (PBS), Alb-Cit, or Alb-MAA (starting at 500 μg/well). These samples were then transferred to coated plates that had been washed 3 times with PBS–Tween, and incubated at 37°C for 45 minutes. Plates were washed in PBS–Tween and a secondary antibody (HRP-conjugated goat anti-rabbit IgG for anti-MAA, HRP-conjugated rabbit anti-mouse IgM [5μ Fc fragment–specific] for anticitrulline) or HRP-conjugated goat anti-human IgG was added (1:2,000), followed by incubation at 37°C for 45 minutes. The plates were washed and TMB substrate was added. Color changes were monitored on an MRX II microplate reader (Dynatech) at 450 nm. The results, expressed as the mean percentage of activity in the 5 samples in the presence or absence of inhibition, were calculated following subtraction of the values for nonspecific activity in incubations with unmodified Alb, as follows: % activity = (OD in the presence of the inhibitor/OD in the absence of the inhibitor) $\times$ 100.

Additional experiments were undertaken to evaluate whether the anti-MAA and/or anticitrulline antibodies reacted with the standard anti-CCP antibody test. The rabbit anti-MAA and mouse anticitrulline antibodies were tested using a Diastat human anti-CCP antibody kit (Axis-Shield Diagnostics). For these studies, the kit was run according to the manufacturer's directions, with the following changes. Rabbit anti-MAA and mouse anticitrulline antibodies were diluted 1:1,000 and substituted for human serum. Plates were washed in PBS–Tween, and a secondary antibody (HRP-conjugated goat anti-rabbit IgG for anti-MAA, HRP-conjugated rabbit anti-mouse IgM [5μ Fc fragment–specific] for anticitrulline) was added (each diluted 1:2,000) to the appropriate wells, followed by incubation at 37°C for 45 minutes. The plates were developed and color change was measured on an MRX II microplate reader. To maintain consistency in the kit, the human standard was used to determine the relative concentration of the anti-CCP antibodies (21,30).

Measurement of antigen-specific ACPAs. Serum samples from RA patients were evaluated for 19 specific ACPAs, in addition to native (unmodified) fibrinogen, using a bead-based multiplex antigen array on the BioPlex platform, as

previously reported (31,32). This array measures disease-specific autoantibody reactivity to multiple citrullinated autoantigens. To assess whether immune responses to MAA are associated with the magnitude of epitope spreading, we examined the number of ACPA-positive analytes, with the threshold for positivity defined as 2 SD above the mean value for all RA patients. We then calculated an ACPA score, defined as the sum of normalized fluorescence values divided by the number of analytes examined (19 ACPAs).

Statistical analysis. Descriptive statistics are shown as the mean ± SD or the frequency with corresponding percentage. Given the skewed distribution of the values, anti-MAA antibody isotypes were log-transformed. To account for confounding from sociodemographic features and health behaviors in the comparisons of anti-MAA antibody isotypes, RA patients were matched to available healthy control subjects at a case:control ratio of 1:1, on the basis of age (±5 years), sex, race, and smoking status. There were 2 controls for which a suitable match to an RA patient was not available, limiting the comparison to 80 patients and 80 controls. A 2-sample *t*-test was used to compare log-transformed anti-MAA isotype concentrations by case and control status. Spearman's correlation coefficients were calculated to examine associations of the different isotype values in RA patients. The associations of anti-MAA isotype concentrations with RA patient characteristics were then examined using unadjusted ordinary least squares regression.

To further examine whether the relationships between anti-MAA isotype concentrations and positivity for RF and anti-CCP antibodies were independent of other covariates, we also performed multivariate ordinary least squares regression analyses with inclusion of all factors that were significant ($P < 0.05$) at the univariate level. Given the high expected concordance between an RF-positive status and anti-CCP–positive status in the RA patients, models including the RF status excluded the anti-CCP status, and models including the anti-CCP status excluded the RF status. Given the skewed distribution of the data, joint counts for regression models were dichotomized into 0 tender or swollen joints and ≥1 tender or swollen joints, with comparisons examining the impact of having a joint count >0. Continuous joint counts are reported for descriptive purposes. Associations of anti-MAA antibody concentrations with clinical measures in these analyses are presented as beta coefficients with corresponding *P* values. The beta coefficient represents the mean log change in anti-MAA antibody concentration associated with a unit change in a given clinical variable.

Significance Analysis of Microarrays (SAM) (32,33) was used to analyze multiplex ACPAs among RA patients who were positive for anti-CCP antibodies, to identify differences in specific ACPA profiles associated with anti-MAA isotype concentrations, categorized into tertiles. We then compared the number of ACPA-positive analytes in addition to ACPA scores based on quintiles of anti-MAA antibody concentrations using one-way analysis of variance, before proceeding to multivariable analyses using analysis of covariance. Covariates in these models included sociodemographic and health-related factors, in addition to RA disease duration. The results of SAM analyses were sorted based on false discovery rates, in order to identify antigens with the greatest differences in autoantibody reactivity. Hierarchical clustering software (Java Cluster version 3.0) was used to arrange the SAM results according to similarities among autoantibody specificities, with SAM scores displayed as a heatmap using Java Treeview software (version 1.1.3). Additional analyses were completed using the Stata (version 12; StataCorp) and SAS (version 9.3) programs.

RESULTS

Synovial tissue samples from 3 RA patients and 3 OA patients were stained for the presence of MAA-modified and citrullinated proteins. As indicated in Figure 1A (blue staining of a representative sample from an RA patient), MAA-modified proteins were detected

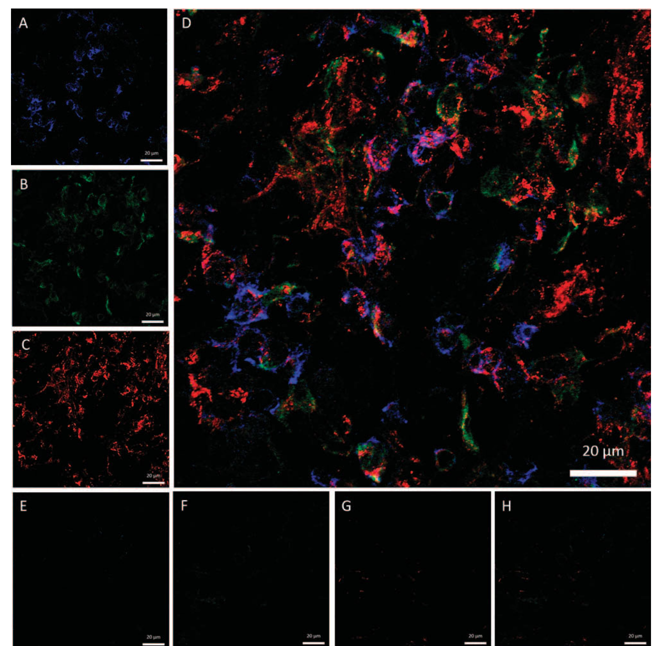

Figure 1. Confocal colocalization images of malondialdehyde-acetaldehyde (MAA) and citrullinated proteins in synovial joint tissue from patients with rheumatoid arthritis (RA) and patients with osteoarthritis (OA). Frozen joint sections from 3 RA patients and 3 OA patients were stained for the presence of MAA, citrullinated proteins, and CD45 using an IgG polyclonal rabbit anti-MAA antibody, IgM monoclonal mouse anticitrulline antibody, and IgG2b anti-rat CD45 antibody. **A,** RA sample stained for MAA proteins. **B,** RA sample stained for citrullinated protein. **C,** RA sample stained for CD45. **D,** Merged image of an RA sample showing MAA proteins, citrullinated proteins, and CD45. **E,** OA sample stained for MAA proteins. **F,** OA sample stained for citrullinated protein. **G,** OA sample stained for CD45. **H,** Merged image of an OA sample showing MAA proteins, citrullinated proteins, and CD45. Images were created using a Zeiss 510 Meta confocal laser scanning microscope and analyzed using Zen 2012 software. Representative images from 1 RA and 1 OA patient are shown (see Supplementary Figure 1 for images from an additional 2 RA patients and 2 OA patients). Original magnification × 63.

Table 1. Reactivity and specificity of monoclonal anti-Cit antibodies, polyclonal anti-MAA antibodies, and RA serum to selected antigens*

	Monoclonal anti-Cit			Polyclonal anti-MAA			RA serum: Anti-MAA+/anti-Cit−			RA serum: Anti-MAA−/anti-Cit+			RA serum: Anti-MAA+/anti-Cit+		
Antigen	No inhibitor	Alb-Cit inhibitor	Alb-MAA inhibitor	No inhibitor	Alb-Cit inhibitor	Alb-MAA inhibitor	No inhibitor	Alb-Cit inhibitor	Alb-MAA inhibitor	No inhibitor	Alb-Cit inhibitor	Alb-MAA inhibitor	No inhibitor	Alb-Cit inhibitor	Alb-MAA inhibitor
Alb-Cit	100	0.8†	99	NA	NA	NA	NA	NA	NA	100	1.1†	99	100	0.8†	100
Alb-MAA	NA	NA	NA	100	100	1.8†	100	99	1.5†	NA	NA	NA	100	99	1.0
Alb-Cit-MAA	100	1.2†	99	100	99	1.4†	100	100	1.1†	100	0.9†	1.0†	100	49†	48†

* For inhibition assays, the monoclonal anticitrulline (anti-Cit) antibody, polyclonal anti–malondialdehyde-acetaldehyde (anti-MAA) antibody, and 5 rheumatoid arthritis (RA) patient serum samples from each of 3 groups (anti-MAA+/anti-Cit−, anti-MAA−/anti-Cit+, and anti-MAA+/anti-Cit+) were assayed for reactivity to citrullinated human serum albumin (Alb-Cit), MAA-modified human serum albumin (Alb-MAA), and human serum albumin citrullinated and then MAA modified (Alb-Cit-MAA). Values are the mean percentage of activity in the presence or absence of inhibition, following subtraction of the values for nonspecific activity in response to unmodified human serum albumin, calculated as (OD in the presence of the inhibitor/OD in the absence of the inhibitor) $\times$ 100. The monoclonal anti-Cit antibody, but not the polyclonal anti-MAA antibody, was reactive to the commercial cyclic citrullinated peptide (CCP) antigen (Diastat). Anti-Cit–positive RA patients were all seropositive for anti–citrullinated protein antibodies (ACPAs) by the anti-CCP test, and all anti-Cit–negative patients were ACPA negative by the anti-CCP test. NA = no or negligible activity of the antibody/serum on the indicated antigen at an OD of $<$0.053, which was determined to be 2 SD above the mean value in negative controls.

† Reactivity was significantly lower ($P < 0.05$) following incubation with the indicated inhibitor.

Table 2. Characteristics of the patients with RA (n = 1,720) at the time of enrollment, and unadjusted associations with anti-MAA antibody isotype concentrations*

Characteristics of the RA patients		Anti-MAA antibody isotype log concentration†		
		IgA	IgM	IgG
Age, mean ± SD years	63.4 ± 11.3	−0.001 (0.637)	−0.006 (0.147)	−0.002 (0.395)
Male, %	90.9	0.267 (0.048)	0.014 (0.928)	0.039 (0.713)
Race, %				
Caucasian	77.5	Referent	Referent	Referent
African American	16.3	0.280 (0.001)	0.056 (0.662)	0.198 (0.040)
Other	6.2	0.375 (0.015)	0.164 (0.418)	0.405 (0.001)
Health-related factors				
Body mass index, mean ± SD kg/m^2	28.2 ± 5.6	0.011 (0.118)	−0.005 (0.561)	0.001 (0.890)
Smoking status, %				
Never	20.2	Referent	Referent	Referent
Former	53.4	0.115 (0.171)	0.023 (0.835)	0.021 (0.814)
Current	26.4	0.175 (0.078)	0.322 (0.011)	0.067 (0.497)
Hypertension, %	57.1	0.133 (0.054)	0.069 (0.467)	0.108 (0.120)
Diabetes mellitus, %	20.7	0.157 (0.052)	−0.161 (0.172)	−0.009 (0.921)
Hyperlipidemia, %	43.2	0.066 (0.329)	0.072 (0.447)	0.167 (0.013)
Cardiovascular disease, %	21.6	0.012 (0.878)	−0.032 (0.793)	0.182 (0.012)
RA-related measures				
Disease duration, mean ± SD years	12.4 ± 11.5	0.005 (0.059)	0.009 (0.009)	−0.001 (0.745)
Nodules, %	29.7	0.205 (0.003)	0.409 (<0.001)	0.251 (0.001)
RF positive, %	79.8	0.351 (<0.001)	0.614 (<0.001)	0.335 (<0.001)
Anti-CCP antibody positive, %	77.9	0.417 (<0.001)	0.408 (<0.001)	0.399 (<0.001)
Swollen joint count, mean ± SD	5.1 ± 6.1	0.108 (0.148)	−0.040 (0.694)	0.086 (0.249)
Tender joint count, mean ± SD	5.8 ± 7.1	0.034 (0.642)	−0.057 (0.566)	−0.036 (0.611)
DAS28, mean ± SD	3.9 ± 1.6	0.068 (0.001)	0.051 (0.066)	0.006 (0.771)
ESR, mean ± SD mm/hour	27.5 ± 23.6	0.206 (<0.001)	0.146 (0.001)	0.171 (<0.001)
CRP positive, %	68.5	0.104 (<0.001)	0.040 (0.262)	0.067 (0.015)
Prednisone use, %	41.8	0.019 (0.783)	0.184 (0.053)	0.045 (0.516)
Methotrexate use, %	51.6	0.023 (0.733)	0.089 (0.345)	0.012 (0.866)
Biologics use, %	22.9	0.154 (0.055)	0.293 (0.010)	0.076 (0.297)

* Models evaluated cross-sectional associations between rheumatoid arthritis (RA) patient characteristics (independent variables) and anti–malondialdehyde-acetaldehyde (anti-MAA) antibody isotype concentrations (log-transformed for analysis) (dependent variables). Joint counts are modeled as a dichotomous indicator (joint count 0 versus >0). The erythrocyte sedimentation rate (ESR) was also log-transformed for analysis. RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; DAS28 = Disease Activity Score in 28 joints; CRP = C-reactive protein.
† Values are the beta coefficient (*P* value).

in the RA synovial tissue. Citrullinated proteins (green staining; Figure 1B) and CD45 (red staining; Figure 1C) were also detected. When these images were merged, independent localization and colocalization of all 3 proteins were evident (purple staining; Figure 1D). Assessment of the OA synovial tissue (Figures 1E–H; representative sample from an OA patient) revealed negligible staining for MAA, citrullinated proteins, and CD45 (Figures 1E–G), and the merged images (Figure 1H) demonstrated no evidence of colocalization of MAA and citrullinated proteins. Similar staining results were observed in the synovial tissue from 2 additional RA patients and 2 additional OA patients (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38969/abstract). Of note, the intensity of staining for MAA (mean ± SD pixel density 16.2 ± 4.8 pixels per inch [ppi] in RA versus 0.7 ± 0.2 ppi in OA), citrullinated proteins (9.6 ± 4.0 ppi in RA versus 1.2 ± 1.0 ppi in OA), and CD45 (10.4 ± 1.7 ppi in RA versus 1.0 ± 0.3 ppi in OA) was consistently higher in RA synovial tissue compared to OA synovial tissue (each n = 3).

To evaluate the specificities of the ACPAs and anti-MAA antibodies, cross-reactivity between the anti-MAA and ACPA assays was evaluated. As shown in Table 1, there was no evidence of cross-reactivity between antibodies to MAA and antibodies to citrullinated proteins. In summary, these data convincingly demonstrated that MAA and citrullinated epitopes represent distinct antibody targets.

In subsequent serologic analyses, serum samples from 1,720 RA patients and 80 healthy volunteer controls were assessed. RA patients included predominantly men (91%) and older patients (mean age 63 years), with the majority of patients reporting Caucasian race (78%)

(Table 2). On average, RA patients had longstanding disease, were predominantly seropositive (78% positive for anti-CCP antibodies), and had moderate disease activity as reflected by the DAS28 (mean ± SD 3.9 ± 1.6). There were moderate and statistically significant correlations between individual anti-MAA isotype concentrations among the RA patients ($r = 0.48–0.58$, $P < 0.001$).

We compared anti-MAA antibody isotype concentrations among 80 randomly selected RA patients and 80 controls matched for age, sex, race, and smoking status. As expected, there were no differences between patients and controls in age (each mean age 51 years), sex (each 78% women), race (each 90% Caucasians), or smoking (each 41% ever smokers). As shown in Figure 2, the log-transformed concentrations of anti-MAA antibody isotypes (IgA, IgG, and IgM) were universally higher among RA patients compared to controls ($P < 0.001$ for all comparisons), regardless of whether patients were positive or negative for anti-CCP.

In analyses defining isotype positivity as a concentration ≥2.5 SD above the mean value in controls, we examined the frequency of anti-MAA antibody isotype positivity in all RA patients with available data (n = 1,720). The prevalence of anti-MAA antibody positivity for each isotype was consistently higher among anti-CCP–positive RA patients compared to anti-CCP–negative RA patients (76% versus 63% for IgA [$P < 0.001$]; 93% versus 88% for IgG [$P = 0.003$]; 41% versus 29% for IgM [$P < 0.001$]). Positivity for all 3 anti-MAA antibody isotypes was observed in 38% of anti-CCP–positive RA patients and 26% of anti-CCP–negative RA patients, compared to none of the control subjects.

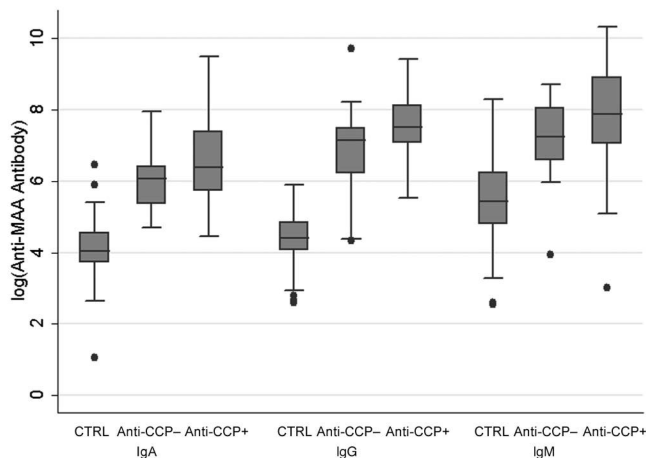

Figure 2. Differences in anti–malondialdehyde-acetaldehyde (anti-MAA) antibody isotype concentrations between rheumatoid arthritis (RA) patients and healthy controls. Controls (n = 80) were matched to RA patients (n = 80) based on age, sex, race, and smoking status, with RA patients stratified as anti–cyclic citrullinated peptide (anti-CCP) positive or negative. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. Symbols represent outliers.

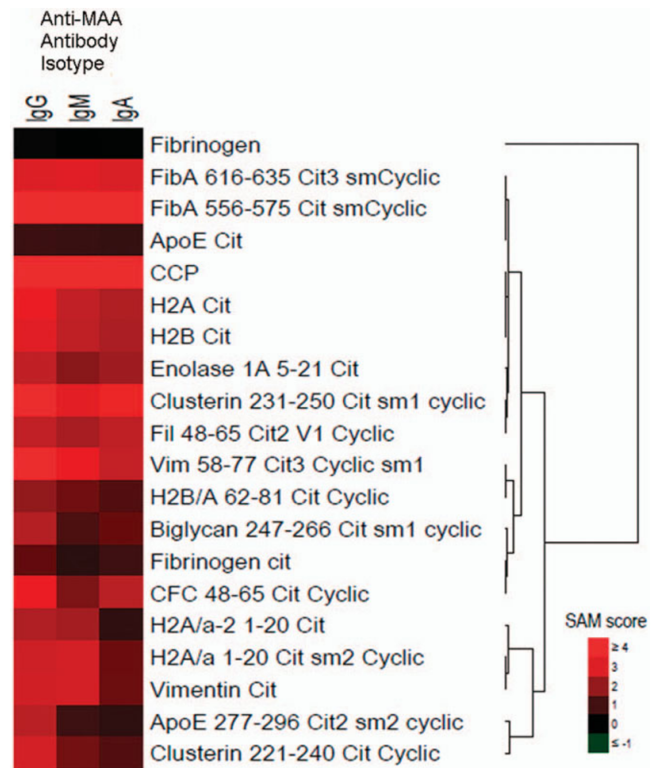

Figure 3. Heatmap demonstrating relative expression of 19 antigen-specific anti–citrullinated protein antibodies (ACPAs) and antibodies to unmodified fibrinogen among rheumatoid arthritis (RA) patients who were positive for anti–cyclic citrullinated peptide (anti-CCP) antibodies. Results reflect Statistical Analysis of Microarray (SAM) scores among those with the highest tertile relative to lowest tertile of anti–malondialdehyde-acetaldehyde (anti-MAA) antibody isotype (IgG, IgM, IgA) concentrations. The heatmap represents the mean expression of antigen-specific ACPAs in the synovial tissue from all 1,720 RA patients analyzed separately for each anti-MAA antibody isotype. FibA = fibrinogen Aα-chain; Apo E = apolipoprotein E; Fil = filaggrin; Vim = vimentin; CFC = filaggrin 48–65 Cit2v1 Cyclic.

Cross-sectional associations of anti-MAA antibody isotype concentrations with RA patient factors are summarized in Table 2. Of note, both the IgA and IgG anti-MAA isotypes were found in higher concentrations among non-Caucasians than among Caucasians. Only the IgA isotype was found at higher levels among men. Compared to never smoking, current smoking was asso-

ciated with higher serum concentrations of the IgM anti-MAA isotype, but not the IgA or IgG isotypes. The concentrations of both the IgA and IgM anti-MAA isotypes were increased with every added year of RA disease duration. In contrast, both hyperlipidemia and cardiovascular disease were associated with higher circulating levels of only the IgG anti-MAA isotype. With regard to measures of disease activity and severity, all 3 isotypes were significantly associated with the presence of subcutaneous nodules, autoantibody positivity (both RF and anti-CCP antibodies), and the ESR.

To further examine whether the associations of RF and anti-CCP antibody status with anti-MAA antibody concentrations were independent of other factors, we examined these associations using multivariate regression analysis. Following multivariate adjustment, RF positivity remained significantly associated with all 3 anti-MAA antibody isotypes ($P \leq 0.006$). In separate multivariable models, anti-CCP antibody positivity remained significantly associated with both IgA ($P = 0.004$) and IgG ($P = 0.001$) anti-MAA isotypes, whereas its association with IgM ($P = 0.11$) was attenuated and no longer significant (data not shown).

Given the significant associations of the anti-MAA antibody isotypes with anti-CCP antibody positivity at baseline and previous data showing associations with tolerance loss (7,21), we examined the associations of these isotypes with the expression of antigen-specific ACPAs, the number of positive ACPA analytes, and the total ACPA score. Multiple ACPAs recognizing several distinct citrullinated antigens were overexpressed among those RA patients with higher anti-MAA antibody concentrations; these findings were consistent across the isotypes, as demonstrated in the heatmap shown in Figure 3. Of the 19 antigen-specific ACPAs examined (including anti-CCP antibodies), 13 demonstrated increased expression that was associated with a higher concentration of one or more anti-MAA antibody isotypes. The number of positive ACPA analytes and the total ACPA score were each significantly higher ($P < 0.001$ for each) among anti-CCP antibody–positive RA patients with higher concentrations of the circulating IgG anti-MAA antibody isotype (Figure 4). Similar results were observed with regard to both the IgA and IgM anti-MAA isotypes (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38969/abstract). The association of all 3 anti-MAA antibody isotypes with the number of positive ACPA analytes and total ACPA score remained significant following multivariable adjustment (data not shown).

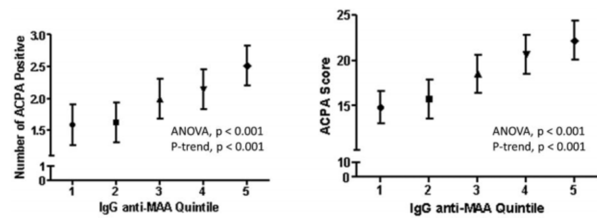

Figure 4. Number of positive anti–citrullinated protein antibody (ACPA) analytes (left) and total ACPA score (right) based on the quintile of circulating IgG anti–malondialdehyde-acetaldehyde (anti-MAA) antibody concentrations in rheumatoid arthritis (RA) patients. The threshold for positivity was defined as 2 SD above the mean value in RA patients. The ACPA score was defined as the sum of normalized fluorescence values divided by the number of analytes examined. Bars show the mean ± SD. ANOVA = analysis of variance.

DISCUSSION

In this study, we found that MAA adducts are present to a greater extent in RA synovium relative to synovium from patients with OA. MAA adducts independently localize and, more importantly, colocalize with citrullinated proteins in the inflamed synovial tissue of RA patients, but not in the synovial tissue of OA patients. For the first time, we have demonstrated that anti-MAA antibody isotype responses are strongly correlated with ACPA seropositivity in patients with RA. Taken together, these data suggest that MAA modifications to RA-related neoantigens could play an important role in tolerance loss and the subsequent generation of immune responses in RA.

Similar to posttranslational deimidation (citrullination) (34), MAA modification of antigens is, in itself, not specific to RA. MAA adduct formation has now been demonstrated in numerous inflammatory disease states, including cardiovascular disease, alcoholic liver disease, and smoking-related lung injury (1,2,4,35). In addition to being overexpressed in the diseased synovium of patients with RA, MAA has been preliminarily demonstrated in atherosclerotic plaque, and antibody isotype responses to MAA have been found to correlate with the progression of cardiovascular disease in non-RA populations (36).

Whether the presence of MAA adducts and increased formation of anti-MAA antibodies could explain, in part, the excess burden of cardiovascular disease in RA is unknown. Speculation in this regard is supported by the higher concentrations of IgG antibodies to MAA observed among RA patients with known cardiovascular disease, as well as higher IgM anti-MAA antibody concentrations observed among cur-

rent smokers, a potent risk factor for heart disease. Cigarette smoke contains AA, a key substrate in MAA formation (1). Although this is beyond the scope of this study and the available data, the question of whether MAA and immune responses to MAA could be used to predict future RA-related cardiovascular events warrants further investigation.

Several questions remain regarding the putative role of MAA in RA pathogenesis. The specific proteins or peptides targeted for MAA modification in RA are, at this point in time, unknown. MAA adducts are formed predominantly on epsilon amino lysine groups and also on select N-terminal amino groups (8), both of which are ubiquitous in human tissue. In alcoholic liver disease, MAA adduct formation stimulates the release of fibronectin and collagen (37), also expressed in articular tissue. Whether MAA adduction specifically targets these peptides or the same peptides undergoing citrullination in RA, or whether these peptides might explain the colocalization of MAA and citrulline in RA synovium observed in this study is unknown and will require further investigation.

Targeted experiments examining the impact of MAA modification on the immunogenicity of citrullinated autoantigens, such as citrullinated forms of vimentin, fibrinogen, or enolase, will be of interest. Given the association of anti-MAA antibodies with increased expression of numerous antigen-specific ACPAs, our results suggest that multiple proteins may be involved. In addition to enhancing immunogenicity, exposure to MAA adducts has also been shown to be cytotoxic (38,39). It is unknown, however, whether immune responses to MAA are directly pathogenic or simply serve as a surrogate marker for its expression.

In addition to the observed associations with anti-CCP antibody positivity, anti-MAA antibody isotypes were detected in 29–88% of anti-CCP antibody–negative RA patients. These results are intriguing and suggest that anti-MAA antibodies could serve as an informative biomarker in seronegative RA. However, these observations must be interpreted with caution given the ubiquitous nature of MAA adduct formation in the context of inflammation and resulting oxidative stress. The potential use of anti-MAA antibodies as either a diagnostic or prognostic biomarker in seronegative RA will require additional studies with the inclusion of disease controls and long-term followup.

Although it would be ideal to directly measure circulating MAA adducts, prior investigations have demonstrated that serum levels of MAA-modified proteins are not readily detectable, most likely because of the high concentrations of circulating anti-MAA antibodies present, in addition to the rapid removal by the reticuloendothelial system (40). There are additional limitations to this study. The RA synovium and serum samples available for this study came from patients with longstanding disease, precluding our ability to examine the possible role of MAA modification and anti-MAA antibody responses earlier in the disease process. Given the very limited number of synovial tissue samples examined, additional studies using a larger number of samples, including those from anti-CCP antibody–negative RA patients and patients with earlier disease, would be of interest.

These data add to a growing body of literature (1,2,4,35) detailing a paradigm of MAA adduction and immune sensitization that can be either functional (i.e., facilitating clearance) or dysfunctional, with MAA-induced dysfunction arising from impaired protein function, early cell death, or, as possible in the case of RA, the promotion of tolerance loss and autoimmunity. When injected into animals, MAA-adducted self proteins trigger robust immune responses even in the absence of adjuvant, with antibody development targeting not only the MAA adduct, but also the carrier protein and the protein carrier conjugate (7,21). Animal studies have demonstrated that the substrates of MAA adduction and MAA adducts are cleared by cells expressing various scavenger receptors (41). Impaired expression or function of these receptors has been implicated in other disease states, leading to MAA adduct accumulation, recruitment and activation of other immune cells, and the increased expression of proinflammatory cytokines and endothelial adhesion molecules (41). Whether these same pathways are also operative in the link between MAA formation and RA remains to be determined.

In summary, we have shown that MAA adduct formation is increased in RA synovial tissue and, importantly, these adducts colocalize with citrullinated antigen. In turn, MAA antibody responses are associated with ACPA expression independent of the multiple covariates examined. In light of these results, future investigations are warranted to identify the role that MAA adduct formation might play in the pathogenesis of RA and the promotion of tolerance loss leading to disease-specific autoantibody formation.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Thiele 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 conception and design. Thiele, Duryee, Anderson, Klassen, Benissan-Messan, O'Dell, Mikuls.

Acquisition of data. Thiele, Duryee, Mohring, Young, Benissan-Messan, Dusad, Hunter, Sokolove, Mikuls.

Analysis and interpretation of data. Thiele, Duryee, Klassen, Sayles, Dusad, Hunter, Sokolove, Robinson, O'Dell, Nicholas, Tuma, Mikuls.

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Applications Invited for *Arthritis Care & Research* Editor (2016–2021 Term)

The American College of Rheumatology Committee on Journal Publications announces the search for the position of Editor, *Arthritis Care & Research*. The official term of the next *Arthritis Care & Research* editorship is July 1, 2016–June 30, 2021; however, some of the duties of the new Editor will begin during a transition period starting April 1, 2016. ARHP or ACR members who are considering applying should submit a non-binding letter of intent by April 15, 2015 to the Managing Editor, Nancy Parker, at nparker@rheumatology.org, and are also encouraged to contact the current Editor, Dr. Marian T. Hannan, to discuss details; initial contact should be made via e-mail to hannan@hsl.harvard.edu. Applications will be due June 15, 2015 and will be reviewed during the summer of 2015. Application materials will be available on the ACR web site.