Production of Autoantibodies against Citrullinated Antigens/Peptides by Human B Cells

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Autoantibodies against citrullinated protein Ags (ACPA) are associated with the development of rheumatoid arthritis (RA). This immune response against citrullinated protein Ags, which is thought to be facilitated by certain MHC HLA-DR alleles, is highly specific for this disease and has been speculated to be involved in the pathogenesis. We have previously studied cultures of B cells for the production of Abs against HLA Ags. The aim of the current study was to examine the role of B cells in the production of ACPA in patients with RA. Peripheral blood B cells from RA patients and healthy people were cultured with EL4-B5, a murine cell line expressing human CD40L, and with T cell factors to stimulate the in vitro production of Abs by B cells isolated from peripheral blood. ACPA were produced by cultured B cells from RA patients, as determined by reactivity to cyclic citrullinated peptide (CCP). The results showed that 22% of the healthy persons tested also had B cells that could produce ACPA. Patients with HLA-DR alleles carrying the RA-associated shared epitope appeared to have more B cells with autoimmune potential for CCP than those without such HLA alleles (odds ratio 8.1, p = 0.001). In healthy individuals, anti-CCP–producing B cells were also observed more frequently if the RA-associated MHC genes were present (odds ratio 8.0, p = 0.01). Analysis of B cells in cultures may shed light on the interaction of genetic and environmental factors in the development of RA.

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The production of autoantibodies against citrullinated protein Ags (ACPA) is a unique marker for rheumatoid arthritis (RA) (1–6). These Abs are present in the majority of RA patients, are virtually never observed in healthy people, and are only rarely found in persons with other conditions (7). Testing for Abs against citrullinated peptides is useful for establishing the diagnosis in patients with early RA (1, 2, 8, 9). Moreover, it is now known that such Abs can be detected some time before the development of clinical disease (10). Both Abs and citrullinated proteins have been observed in affected joints (11), and immune complexes consisting of citrulline-specific Abs and citrullinated fibrinogen have been found in the blood of patients with RA (12) and are likely to play a role in the pathogenesis of RA-associated vasculitis.

The presence of ACPA has been associated with the known RA-associated MHC genes (13) and with certain environmental factors such as the use of tobacco and exposure to cigarette smoke (14, 15). There is evidence that conversion of arginine to citrulline at the peptide side-chain position of vimentin and human proteoglycan aggrecan, interacting with the shared epitope, significantly increases peptide-MHC–binding affinity, and in one experiment, appears to have led to the activation of CD4+ T cells in DR4-transgenic mice (16). These results have been interpreted to suggest that DRB1 alleles with the RA-associated shared MHC epitopes might facilitate an immune response to citrullinated proteins and lead to the production of Abs that characterize the development of RA (14). In addition, DR4-transgenic mice, when immunized with citrullinated fibrinogen, were found to develop arthritis (17), a condition that was transferable to naive recipients by T cells from immunized animals (18) and could be suppressed by treatment with CTLA-4Ig (18).

Not much is known about the role of B cells in this autoimmune response. We have previously developed and studied an assay for Ab production against HLA Ags by human peripheral blood B cells in tissue culture (19). It seemed possible that a similar culture system might work also for the study of the production of ACPA. Therefore, in the present experiments, B cells from patients with RA and normal human subjects were cultured to test for the production of Abs against cyclic citrullinated peptide (CCP). Having shown that autoantibody production can be detected, we have used this method to evaluate the role of the presence of the RA-associated shared HLA-DR epitopes, and the effect of smoking, on the production of ACPA in RA patients and in normal subjects.

Materials and Methods

Patients

Included in this study were 66 patients with RA (58 women, 8 men, ages ranging from 23 to 79 y, mean 54 y) and 63 healthy persons without any personal or family history of RA (42 women, 21 men, ages ranging from 22 to 69 y, mean 41 y) who were rheumatoid factor (RF) and anti-CCP negative. The patients met the American College of Rheumatology classification criteria for RA (20). Clinical and laboratory information was obtained from medical charts and included age, sex, disease duration, smoking history, RF assay, and results of anti-CCP Ab testing. RA patients had a disease duration ranging from 1 to 42 y, and therapy included disease-modifying antirheumatic drugs, biological agents, and glucocorticoids. Among the RA patients, 42 (63.6%) had smoked at some time and 8 (22.2%) were current smokers. In the normal subjects, 12 (19.0%) were former smokers and 8 (12.7%) were smoking at the time of the study. The study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center, and informed consent was obtained from each participant.
EL4-B5, a murine cell line expressing human CD40L, was obtained from J.E. Crow (Vanderbilt University) with permission from the cell line developer R.H. Zubler (Hoptal Cantonal Universitaire, Geneva, Switzerland).

Isolation of human B lymphocytes and CD27+ memory B cells

Samples of 30–40 ml citrate dextrose-treated peripheral blood were obtained from patients and normal subjects. PBMCs were obtained by Ficoll-amidotrizoate centrifugation, and B cells were isolated by negative selection using a B cell isolation kit that removes cells positive for CD2, CD14, CD16, CD36, CD43, and CD235a and a MACS column type LS (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The number of B cells obtained in each experiment varied between 0.8 and 3.0 x 10^6. The highly enriched population of B cells was analyzed by flow cytometry using a PE-conjugated mAb against CD19 (BD Pharmingen, San Diego, CA) and a PerCP-conjugated mAb against CD3 (BD Pharmingen). The purity of the B cell preparations was found to be 85–90%. In some experiments, the B cell population was further fractionated using anti-CD27–coupled microbeads (Miltenyi Biotec) into CD27 positive and CD27 negative subsets with anti-CD27–conjugated magnetic beads or into CD19+CD5+ and CD19+CD5- preparations of B cells using anti-CD5 PE-conjugated Ab to attach to the cells and isolation of the CD5+ cells with anti-PE microbeads.

B cell cultures

Peripheral blood B cells (1 x 10^6/well) were cultured in 48-well plates with a feeder layer of irradiated (50 Gy) EL4-B5 (2 x 10^5/well). The cell cultures were incubated at 37°C in humidified atmosphere with 5% CO2. The cell culture medium used was RPMI 1640 supplemented with 10% FCS, 2 mm L-glutamine, 0.05 mg/ml gentamycin, 5 mg/ml PMA (Sigma-Aldrich), and 5% T cell culture supernatant. T cell supernatant was produced by cultivating enriched T lymphocytes (Rosette-Sep; StemCell Technologies, Vancouver, BC) from a healthy male donor for 36 h in the presence of 5 μg/ml anti-CD3 mAb (Sigal-Aldrich) and 10 ng/ml PMA. After 7 d of incubation, B cells were restimulated with addition of EL4-B5 cells (2 x 10^5/well), 5 μg/ml PMA, and 5% of T cell supernatant. The B cell supernatants were harvested on day 14 of culture.

ELISA for detection of Abs against CCP3 and cyclic arginine peptide 3

B cell culture supernatants, taken from individual wells, were concentrated five times using UltraTec YM-10 Centricron filters (Millipore, Billerica, MA), and were analyzed using commercially available CCP3 ELISA trays or cyclic arginine peptide 3 (CAP3) trays containing peptides with arginine instead of citrulline (Innova Diagnostics, San Diego, CA). In addition, a low positive standard and a negative control consisting of culture medium alone were also used. Concentrated supernatants (100 μl) were incubated in individual wells for 1 h and were washed three times, and then 100 μl biotin-labeled anti-human IgG (1:50,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) was added to each well for 30 min, followed by washing five times. Subsequently, the wells were incubated with 100 μl HRP conjugated with streptavidin (1:50,000 dilution; Jackson ImmunoResearch Laboratories) for 30 min. Then, the plates were washed again five times, and color was developed with tetramethylbenzidine (Bethyl Lab, Montgomery, TX). Reactions were stopped with 100 μl 0.344 M sulfuric acid after 10 min, and absorbance was read at 450 nm. In some experiments, CAP3 trays were used to determine whether the Abs detected were specific for citrulline. They were treated identically to the CCP3 trays. Abs were quantitated using a standard curve of sequential dilutions of the low positive standard for both CCP3 and CAP3, and the results were expressed in units based on the standards provided by the manufacturer. cutoff values were determined as the mean plus three times the SD of B cell supernatants obtained from healthy controls (21) and were found to be specific for CCP3 and 10.94 units for CAP3. B cell supernatants were considered negative when all the wells obtained from one person yielded supernatants that did not contain detectable anti-CCP Abs. When one or more supernatants were found to have anti-CCP Abs, the experiment was scored as positive. The strength of the in vitro B cell response was determined as the average of CCP3 units of all the wells tested from each subject and the frequency of positive wells, which represents a fraction of the number of wells with CCP3 units above the cutoff divided by the total number of wells, assayed in each experiment per subject.

Detection of anti-CCP Abs in serum

Serum samples were also collected from both populations and tested for detection of anti-CCP3 Abs using CCP3 ELISA kit (Inova Diagnostics).

HLA typing and PTPN22 1858C/T polymorphism

Genomic DNA was extracted from PBMCs using Qiagen DNeasy blood and tissue kits (Qiagen), and HLA-DRB1 alleles were determined by group-specific amplification and sequence-based typing. The following RA-associated HLA-DRB1 alleles were considered as susceptibility alleles: DRB1*01:01, DRB1*01:02, DRB1*01:04, DRB1*04:01, DRB1*04:04, DRB1*04:05, DRB1*04:08, DRB1*04:09, DRB1*04:10, DRB1*04:13, DRB1*04:16, DRB1*10:01, DRB1*14:02, DRB1*14:06, and DRB1*14:09. PTPN22 1858C/T polymorphism (rs2476601) was determined in selected RA patients and healthy subjects who had B cell cultures producing ACA. The PCR was performed according to the instructions provided by the manufacturer, and the genotypes were detected using an ABI PRISM 7900 HT Sequence Detector System (Applied Biosystems, Foster City, CA).

Analyses of cytokines

Serum levels of five cytokines (IL-2, IL-4, IFN-γ, TNF-α, and GM-CSF) were measured using a human cytokine Milliplex map kit (Millipore). The assay was performed according to the protocol, and was collected using a Luminex 100 instrument (Luminex, Austin, TX). To remove potential nonspecific effects in the cytokine assay due to the presence of RF, in parallel experiments, the sera from RA patients were treated with a blocking reagent developed for this purpose (HeteroBlock; Omega Biologicals, Bozeman, MT). HeteroBlock was added to the serum sample to achieve a final concentration of 3 μg/ml, a concentration reported to be optimal for this assay (22). A much higher concentration of this reagent has recently been used by other workers (23), but was not thought necessary in our experiments. Cytokine concentrations were calculated based on a weighted five-parameter logistic curve-fitting method with Masterplex software.

Statistical analysis

Odds ratios (OR) and 95% confidence interval were calculated to investigate the contribution of the RA-associated MHC alleles and smoking to the presence of anti-CCP Abs in B cell cultures. Differences in cytokine levels were analyzed using the Student t test with Welch’s correction when appropriate. Differences in levels of anti-CCP Abs expressed as median of anti-CCP3 units in positive wells or frequency of anti-CCP3-positive wells were analyzed by the Mann–Whitney U test. In all tests, the p values <0.05 were considered significant. GraphPad Prism software was used (GraphPad Software, La Jolla, CA).

Results

Peripheral blood B cells from RA patients produce autoantibodies in vitro

We studied a group of RA patients with typical disease manifestations, including erosions of bone and presence of serum Abs against CCP in 60 of 66 (90.9%) and RF in 62 of 66 (93.9%). Cultures of their peripheral blood B cells with EL4-B5 feeder cells expressing CD40L and added T cell factors produced Abs against CCP in 39 of the 66 patients studied (59.1%) (Table I). Fig. 1 shows the results obtained in each of the experiments with B cells from RA patients. Each data point represents the Abs produced in one well as determined by ELISA with CCP3. In the experiments in which autoantibodies against CCP were not detectable, the cultures were found to be viable and the B cells were producing other Abs because IgG was detected in the supernatants (data not shown). IgG production in these cultures, including RA patients and normal controls, was fairly constant. The mean production of IgG was 55.4 ± 4.0 ng/ml. There was no difference in the amount of IgG produced between cultures of B cells from RA patients and normal subjects. Depending on the yield of B cells, between 5 and 42 wells were available from each person for analysis. The frequency of supernatants with anti-CCP Abs in each patient was variable, from just a few wells to all the wells tested. Anti-CCP Abs
the serum of RA patients did not correlate with the frequency of ACPA-positive wells \((p = 0.7)\). Also, not all patients with anti-CCP detected in the serum produced positive B cell cultures. There were some cultures that produced few or no CCP Ab-containing supernatants that were obtained from patients with high levels of serum anti-CCP of \(>100\) units. The reason for this is not clear.

**Autoantibodies against citrullinated peptides in cultures of B cells from normal subjects**

When B cells from 63 healthy persons were cultured with the EL4-B5 feeder cells and T cell factors, the majority of the cultures (77.8\%) were negative for anti-CCP. However, in a few instances (22.2\%), Abs to CCP3 above the threshold used as cutoff were detectable in the cultures of B cells from normal subjects (Table II). Fig. 2 shows the detail of the production of anti-CCP in B cell cultures from normal subjects. As can be seen, the majority of the cultures from normal subjects were negative, but a few wells had values that were above the threshold. In 12 of these subjects, one to three wells were considered positive. These results suggested that B cells from some people without any known autoimmune condition can produce Abs to CCP under these culture conditions.

**Anti-CCP Ab-producing B cells developed mostly from the CD27-positive CD5-positive subset of B cells in the blood and could be CD38 positive or CD38 negative**

Among circulating human B cells, a CD27-positive subset is considered to represent memory B cells. To determine whether anti-CCP Ab-producing cells originated from naive B cells or from memory B cells, we separated the two populations from preparations of CD19-positive B cells, using anti-CD27–coupled magnetic beads. In five RA patients studied, the majority of the anti-CCP–producing B cells came from the CD27-positive subset. This was similar to what we had previously observed with B cells producing Abs against HLA Ags (19). In three of five experiments, all the wells obtained from CD27-positive B cell preparations produced anti-CCP Abs. In the other experiments, one

### Table I. Association of HLA-DR RA susceptibility alleles with production of Abs against citrullinated peptides in B cell cultures from 66 RA patients

<table>
<thead>
<tr>
<th>RA-Associated MHC Alleles</th>
<th>Number of Patients</th>
<th>Anti-CCP3–Positive Number (%)</th>
<th>Anti-CCP3–Negative Number (%)</th>
<th>Odds Ratios (95% Confidence Interval)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All RA patients</td>
<td>66</td>
<td>39 (59.1)</td>
<td>27 (40.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SE absent</td>
<td>17</td>
<td>4 (23.5)</td>
<td>13 (76.5)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>SE present*</td>
<td>49</td>
<td>35 (71.4)</td>
<td>14 (28.6)</td>
<td>8.1 (2.3–27.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>One copy</td>
<td>29</td>
<td>19 (65.5)</td>
<td>10 (34.5)</td>
<td>6.2 (1.6–22.8)</td>
<td>0.015</td>
</tr>
<tr>
<td>Two copies</td>
<td>20</td>
<td>16 (80.0)</td>
<td>4 (20.0)</td>
<td>13.0 (2.8–60.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*SE present, one or two copies of the RA-associated HLA-DR susceptibility alleles given in Materials and Methods.

**FIGURE 1.** Production of Abs against CCP3 by B cells from 66 RA patients. B cells were isolated and cultured, as described in Materials and Methods. Supernatants from cultures of \(1 \times 10^5\) cells were harvested after 14 d, concentrated five times, and analyzed by ELISA with CCP3 trays. Each data point in the figure represents the result obtained with one culture supernatant. The threshold for positive results was determined from the supernatants obtained from the normal subjects tested as mean plus three times the SD. This cutoff is shown by the horizontal line at the level of 7.88 anti-CCP3 units. Values below the cutoff are shown as circles; results above the cutoff and considered positive are depicted as triangles. Anti-CCP3 production above the threshold was observed in 39 (59.1\%) of the RA patients studied.
was 75% and the other 60%, whereas the frequency of anti-CCP3-positive wells was low in the CD27-negative subpopulation of B cells, with a frequency >20% seen in only one experiment. To determine the contribution of CD19+CD5+ cells, we isolated CD5-negative and CD5-negative B cells from four RA patients prior to culture. After culture, we obtained nine wells producing ACPA; seven of them were CD19+CD5+ and only two were CD19+CD5-2. Thus, 78% of the B cells producing ACPA were of the CD5+ type. This enrichment of CD5+B cells contrasts with the 64 ACPA-negative wells that were derived from CD5+ cells in only 4 (5.9%) cases (p < 0.0001). B cells that are activated to become early plasma cells can be identified by the presence of CD38 on the surface. Therefore, to further characterize the B cells that produce ACPA in these cultures, we separated CD19+B cells into CD38-negative and CD8-negative subsets. These experiments were performed with samples obtained from two RA patients and four healthy controls. Cultures from RA patients produced 19 supernatants that were ACPA positive; 4 (21.0%) came from CD19+CD38+ cells and 15 from CD19+CD38- B cells. In the ACPA-negative cultures, 3 were derived from CD19+CD38+ cells and 23 from CD19+CD38- B cells. In the experiments with cells from healthy persons, we obtained 30 wells that were ACPA positive. Of these, 5 (16.7%) were produced from CD19+CD38+ B cells. Supernatants without ACPA were derived from 19 CD38+ and 53 CD38-CD19+ B cells. In either case, the differences were not significant (p > 0.4). We concluded that in RA patients, as well as in healthy subjects, a fraction of the cultures (21.0 and 16.7%) was derived from activated CD19+CD38+ B cells or early plasma cells.

Quantitation of anti-CCP production in B cell cultures
To study how much anti-CCP was produced in the B cell cultures, we calculated the median of the anti-CCP units as well as the mean and SD of the frequency of positive wells. The median anti-CCP response in cultures of B cells from normal subjects was 1.4 and the frequency 0.05 ± 0.09. Thus, patients with RA produced larger amounts of anti-CCP.

### Table II. Association of HLA-DR RA susceptibility alleles with production of Abs against citrullinated peptides in B cell cultures from 63 normal subjects

<table>
<thead>
<tr>
<th>RA-Associated MHC Alleles</th>
<th>Number of Subjects</th>
<th>Anti-CCP3–Positive Number (%)</th>
<th>Anti-CCP3–Negative Number (%)</th>
<th>Odds Ratios (95% Confidence Interval)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total normal subjects</td>
<td>63</td>
<td>14 (22.2)</td>
<td>49 (77.8)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SE absent</td>
<td>30</td>
<td>2 (6.7)</td>
<td>28 (93.3)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>SE presenta</td>
<td>33</td>
<td>12 (36.4)</td>
<td>21 (63.6)</td>
<td>8.0 (1.8–35.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>One copy</td>
<td>30</td>
<td>11 (36.7)</td>
<td>19 (63.3)</td>
<td>8.1 (1.8–36.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Two copies</td>
<td>3</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
<td>7.0 (0.7–88.5)</td>
<td>0.63</td>
</tr>
</tbody>
</table>

aSE present, one or two copies of the RA-associated MHC susceptibility alleles given in Materials and Methods.

![FIGURE 2](http://www.jimmunol.org/) Production of Abs against CCP3 by B cells from 63 normal subjects. B cells were isolated and cultured, as described in Materials and Methods. Supernatants from cultures of 1 × 10⁵ cells were harvested after 14 d, concentrated five times, and analyzed by ELISA with CCP3 trays. Each data point in the figure represents the result obtained with one culture supernatant. The threshold for positive results was determined from the supernatants obtained from the normal subjects tested as mean plus three times the SD. This cutoff is shown by the horizontal line at the level of 7.88 anti-CCP3 units. Values below the cutoff are shown as circles; results above the cutoff and considered positive are depicted as triangles. The majority of the B cells from normal subjects did not produce anti-CCP3 in vitro. Anti-CCP3 production above the threshold was observed in 14 (22.2%) of the normal subjects studied.
anti-CCP Abs, and the differences were statistically significant ($p < 0.01$ and $p < 0.0001$, respectively).

**Abs against CCP3 detected in B cell cultures were specific for citrullinated peptides**

When peptides containing arginine (CAP3) were used in the ELISAs, instead of the citrullinated peptides, it was found that 4 of 365 supernatants obtained from B cell cultures from 39 RA patients who had positive B cell cultures for anti-CCP were reactive (data not shown). In 88 cultures from 11 normal subjects, only 1 had a CAP3 value above the threshold. The conclusion was that the Abs produced by cultured B cells and detected in the culture supernatants were largely specific for the citrullinated form of the peptides. They were autoantibodies against citrulline found in the substituted peptides. Most of them did not recognize the native arginine-containing peptides.

**RA-associated MHC alleles and production of anti-CCP by cultured peripheral blood B cells**

The HLA class II alleles of patients and normal subjects in this study were determined by sequence-based typing. Among the RA patients, 29 (43.9%) had one copy and 20 (30.3%) had two copies of the RA-associated DRB1 alleles. In the normal subjects, a single copy of an allele having the shared epitope was seen in 30 (47.6%), and two copies were present in only 3 (4.8%). It has been previously suggested by others that the RA-associated MHC alleles, which were found to bind citrullinated proteins/peptides with greater affinity, can facilitate the immune response against citrullinated peptides (13). We therefore analyzed the donors of the B cell cultures for the presence of RA-associated MHC alleles. The results show that presence of a MHC risk allele carrying the shared epitope was associated with more frequent anti-CCP3–positive B cell cultures in RA patients (Table I, OR = 8.1, $p = 0.001$). This was true when anti-CCP3 production was measured as the median of anti-CCP units per culture or as the frequency of anti-CCP–positive wells (Fig. 3). Also, the OR for anti-CCP3–positive B cell cultures was higher in RA patients with two copies of RA-associated MHC alleles (Table I, OR = 13.0) than in patients with a single copy (Table I, OR = 6.2). These results at the cellular level are in agreement with previous observations indicating that production of anti-CCP detected in the serum of patients was associated with the presence of the RA-associated shared epitopes (24). Because it has been reported that the polymorphic marker PTPN22 1858T was associated independently with the presence of anti-CCP Abs in patients with early RA (25), 10 RA patients having many B cells that produced ACPA were typed for this genetic marker. The frequency of 1858T was 33%, not significantly different from a group of RA patients producing very few or no ACPA in vitro. In addition, in 10 healthy persons whose B cell cultures produced ACPA, only 2 carried PTPN22 1858T, indicating that there was no association with this genetic marker.

**FIGURE 3.** Quantitation of the anti-CCP3 response in B cell cultures from 66 RA patients (A, B) and 63 normal subjects (C, D). The number of wells available for analysis depended on the number of B cells obtained and was between 5 and 42 wells for cultures from each person. The results are presented as median of anti-CCP3 units produced in each experiment (A, C) or as the frequency of anti-CCP3–positive wells (B, D). Also shown is the effect of the presence or absence of the RA-associated shared determinant. Presence of the RA-associated MHC alleles was associated with higher mean anti-CCP3 units and higher frequency of anti-CCP3–positive wells in RA patients and in normal controls. The $p$ values were obtained by the Mann–Whitney $U$ test.
Effect of cigarette smoking on B cell cultures from RA patients

Smoking was prevalent among the RA patients studied, which included 42 of the 66 (63.6%) RA patients who had smoked at some time and 18 (27.3%) who were current smokers. Production of anti-CCP in B cell cultures was associated with smoking in these patients. As can be seen in Fig. 4, current smokers produced higher median anti-CCP units ($p < 0.02$) and a trend for greater frequency of positive wells that did not reach statistical significance ($p < 0.08$). Fewer of the healthy subjects studied were smokers, as follows: 20 of 63 or 31.8% compared with 63.6% among the RA patients. A significant association of smoking with median anti-CCP production in B cell cultures of normal subjects was not observed with either former or current smokers. However, when analyzed as the frequency of anti-CCP-positive wells (Fig. 4D), the differences between smokers and nonsmokers were observed to be statistically significant (former smokers, $p < 0.05$; current smokers, $p < 0.05$).

Serum concentration of cytokines in RA patients and in normal subjects whose B cell cultures either produced or did not produce Abs against CCP

Several cytokines and chemokines can be increased in persons who later develop RA (26). At the same time, before the onset of symptoms of RA, some of those persons also may have increased levels of anti-CCP Abs in their serum. Because some of the normal subjects studied in this work had B cell cultures producing anti-CCP in vitro, we wondered whether any of these subjects had increased levels of cytokines. Using a multiplex system, we analyzed the sera from the normal donors of B cells we had studied, to determine the levels of cytokines as shown in Table III. The 61 RA patients included in this study were also tested. Because RF, acting as heterophilic Abs, can affect the results of testing for cytokines (22), we used HeteroBlock (Omega Biologicals) to reduce nonspecific binding (23). As expected, the mean levels of the cytokines studied were quite elevated in this group of patients and were significantly higher than levels observed in the normal subjects studied who had positive B cell cultures producing anti-CCP (Table III). The cytokines investigated showed similar levels in both groups of normal controls. Therefore, the cytokines studied were not elevated in normal persons whose B cells could be stimulated to produce Abs against CCP.

Discussion

The results in this study show that B cells from RA patients can produce IgG anti-CCP Abs in vitro. The threshold used to determine which culture supernatants were positive was determined, as is customary (21), from the normal controls, taking the mean plus three times the SD. In our previous work with Abs against HLA Ags, the specificities of the Abs produced in vitro were similar to those observed in the serum of the same patients. Rarely did we see Abs produced by cultured B cells that were not detectable in the serum. In RA patients, production of anti-CCP in B cell cultures occurred in patients who had anti-CCP in the serum. Most of the B cells producing anti-CCP Abs in cultures originated from CD27-positive memory B cells, and this too was similar to what was previously observed with B cells producing Abs against HLA (19).

In the case of the subjects without RA, 14 of 63 normal subjects tested, who did not have anti-CCP in the serum, produced anti-CCP in the B cell cultures. These normal subjects did not have either a personal or a family history of RA, and their sera were tested and found to be negative for anti-CCP. The amounts of IgG produced were all about the same in patients and normal subjects because the B cell cultures were stimulated with CD40L from the feeder cells and by the addition of T cell factors. These conditions were
identical to those used in the assay for Abs against HLA (19). They were suitable for stimulating production of many different Abs.

We do not know why these normal persons had B cells in the peripheral blood that could produce autoantibodies. It is known that large numbers of B cells with potential for producing autoantibodies are normally produced (27). These potentially harmful cells are either eliminated or silenced by mechanisms that have been studied under various conditions (27). However, Tillot et al. (28) found that normal subjects had many IgG-positive memory B cells that produced autoantibodies that produced nuclear or cytoplasmic staining of HEp-2 cells. These Abs were indistinguishable from those produced by autoantibodies that produced anti-CCP. The results showed that normal subjects had many IgG-positive memory B cells producing anti-CCP in culture. In healthy persons, RA patients, smoking, especially current smoking, increased the frequency of B cells producing ACPA compared with those from non-smoking controls. One can only speculate about what may be the explanation.

In RA patients, the production of Abs against CCP was associated with the presence of certain HLA-DR alleles that have been known for many years to constitute risk factors for the development of RA. More recent analysis based on studies of several cohorts of RA patients (13) has suggested that the MHC genes are associated with the production of anti-CCP rather than with the development of the disease (30, 31). In that context, our results seem to be in agreement. Production of anti-CCP by B cells from RA patients was associated with the presence of the HLA-DR alleles that carry the shared epitope (but not of PTPN22 1858T) and production of anti-CCP in vitro (OR = 8.1, p = 0.001). We could not see an additional effect from the presence of the marker PTPN22 1858T in the small sample examined.

Because memory B cells from some of the normal subjects studied, who did not have and most likely never would develop RA, were found to produce Abs against CCP, we examined whether the HLA-DR alleles that carry the RA-associated shared epitope might also be correlated in this group of subjects. The results indicated that there was an association between presence of HLA-DR with shared epitope (but not of PTPN22 1858T) and production of anti-CCP in vitro (OR = 8.0, p = 0.01). This finding, which is difficult to explain, needs to be confirmed in a larger group of normal subjects. One can only speculate about what may be the explanation. It is tempting to argue that the mechanism of this association could well be similar in RA patients and controls. It is presumed that MHC molecules present the citrullinated proteins/peptides to T cells and that the latter in some way regulate the autoimmune memory B cell response. In either case, an expansion of the IgG positive B cells capable of producing ACPA were already activated in vivo.

Table III. Serum levels of cytokines were elevated in RA patients and not increased in normal subjects

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Anti-CCP-Negative Normal Subjects (n = 46)</th>
<th>Anti-CCP-Positive Normal Subjects (n = 14)</th>
<th>RA Patients (n = 61)</th>
<th>p*</th>
<th>p#</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>1.8 (0.3–36)</td>
<td>4.1 (0.3–22.7)</td>
<td>79.8 (0.3–804.8)</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.3 (0.2–45.7)</td>
<td>0.9 (0.2–4.7)</td>
<td>20.2 (0.1–224.6)</td>
<td>&lt;0.0001</td>
<td>0.0007</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.8 (0.1–1.0)</td>
<td>0.8 (0.1–1.5)</td>
<td>12.0 (0.3–82.8)</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.8 (0.4–3.2)</td>
<td>2.6 (0.4–3.2)</td>
<td>27.1 (0.1–264.1)</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.0 (0.4–10.6)</td>
<td>3.7 (0.4–12.0)</td>
<td>17.0 (0.1–158.4)</td>
<td>0.0001</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

*The p values for comparison of cytokine concentration in serum of normal subjects with negative B cell cultures compared with those with B cell cultures producing anti-CCP.

#The p values for comparison of cytokine concentration in serum of RA patients with negative B cell cultures compared with those with B cell cultures producing anti-CCP.

Results for RA patients obtained before treatment of serum with HeteroBlock.

Results for RA patients obtained after treatment of serum with HeteroBlock.

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were able to produce Abs against HLA Ags in vitro (19, 32). The present results show that B cells from RA patients can produce IgG anti-CCP Abs in vitro under similar conditions. In one previous report, IgM Abs against CCP were found to be produced in B cell cultures (33). We have not tested for IgM anti-CCP in the present experiments. Not all patients with detectable anti-CCP in serum produced cultures of B cells making anti-CCP. These results were not due to culture failure because we determined that those cultures contained active B cells producing IgG and were therefore viable. Some failures were probably due to sampling error, especially if the number of B cells retrieved was small and only a few culture wells could be established. Other factors causing negative cultures could have been the effect of medications taken by the patients or technical problems with the assay that have not been identified.

In RA patients, anti-CCP Abs were associated with elevated levels of cytokines. It has been observed by others that high levels of cytokines (26) and systemic indicators of inflammation (34) develop together with autoantibodies in RA. In normal subjects who had B cells capable of producing anti-CCP in the current study, levels of inflammatory cytokines were not elevated. This combination of data, clinically normal subject with B cell cultures producing anti-CCP, with no autoantibodies or elevation of cytokines in the serum, defines a state that in most cases probably does not evolve to active RA. The absence of inflammatory cytokines suggests that T cell activity, if it exists, is maintained below a critical threshold. Autoimmune B cells, if expanded as detected in our assay, are not active in vivo.

It is not known what ultimately leads to the development of clinical disease in subjects having the known risk factors. Our results suggest that a small number of normal subjects may have B cells capable of making autoantibodies against citrullinated peptides. Such B cells were observed more frequently when the MHC alleles that predispose to the development of RA were also present. Because the HLA class II alleles are recognized by T cells, the role of T cells in the expansion of those B cell clones would be of interest (30).

In the present experiments, inflammatory cytokine levels in normal subjects having B cells that can produce anti-CCP were normal. However, there is evidence that when the events progress toward clinical expression of disease, inflammatory cytokines may be elevated before clinical arthritis is detected (26). Eventually, inflammatory arthritis develops and erosive disease ensues. A number of other genes such as PTPN22 (35), TRAF1/C5 (36), and STAT4 (37) also appear to play a role in this process. In the present experiments, a group of RA patients with strong ACPA production in B cell cultures and some normal subjects with B cell cultures producing ACPA were analyzed for inheritance of the PTPN22 1858T allele. In this limited sample, the PTPN22 allele known to be associated with RA did not appear to increase.

These experiments show that a method initially developed to study autoantibodies can equally well be used for the study of autoantibodies in patients with RA. In many of these cultures, the frequency of anti-CCP–producing B cells was very high and appeared to develop predominantly from CD27-positive memory B cells in the peripheral blood. In comparison with the cultures obtained from RA patients, B cells from normal subjects produced autoantibodies to CCP quite rarely, and the number of such B cells was also much smaller. It is intriguing that the frequency of autoimmune B cells producing anti-CCP was associated with the presence of HLA-DR alleles that have the shared epitope and are known to be associated with this disease. Presence of at least one copy of the HLA-DR–shared epitope was associated in normal subjects with a higher frequency of positive anti-CCP responses. It is not known how this comes about. If confirmed, this result might suggest that the MHC exerts an influence on the pool of autoimmune B cells in healthy persons. It is likely that analysis of autoimmune B cells by the methods described may yield further interesting results in the study of RA and possibly other autoimmune diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


