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Increased Concentrations of Antibody-Bound Circulatory Cell-Free DNA in Rheumatoid Arthritis

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Background: **Increased concentrations of cell-free DNA have been found in several disorders and have been interpreted as evidence of increased rates of cell death or turnover. Evidence from in vitro and animal experiments suggests that DNA may play a role in the pathogenesis of rheumatoid arthritis (RA).**

Methods: **We measured cell-free DNA in plasma and serum from patients with RA and healthy controls by use of quantitative PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) DNA. We used protein G Sepharose™ bead adsorption of plasma and elution to isolate antibody-bound DNA.**

Results: **In paired plasma and serum samples of 16 healthy controls the median *GAPDH* copies were 4500 genome equivalents (GE)/mL plasma (range 319–21 000) and in 26 RA patients 17 000 GE/mL plasma (2100–2 375 000, $P = 0.0001$). In the serum from normal controls the median *GAPDH* copies were 35 000 GE/mL (1700–239 000) and from RA patients 222 000 GE/mL (21 000–2 375 000, $P = 0.004$). A median of 81% of the cell-free DNA in RA was associated with antibody compared with 9% in healthy controls ($P = 0.001$). The concentrations of DNA did not vary with the type of therapy patients received.**

Conclusions: **These results provide new evidence for a role of cell-free DNA-antibody complexes in the etiology of RA, suggest new avenues for basic research, and may prove to be relevant to diagnosis and assessment of therapy.**

Rheumatoid arthritis (RA)[4] is an inflammatory, destructive joint disease in which a broad range of extra-articular manifestations may occur. Although a score of cellular and humoral abnormalities have been characterized, the underlying mechanism of the disease process has remained elusive *(1)*. The pannus, a tissue composed of cells derived from synovial fibroblasts, macrophages, and B and T cells, is involved in the destruction of joint cartilage and adjacent bone. Cytokines, primarily tumor necrosis factor (TNF)-α and interleukin-1, mediate the formation of the pannus and maintain its destructive capacity *(2)*. The remarkable improvement of a large proportion of patients with RA by agents that block TNF-α has underscored the pathophysiological significance of this cytokine in RA *(3, 4)*.

The events precipitating the cytokine release have yet to be identified. Several lines of evidence suggest a direct role of synovial tissue. On the other hand, T and B cell responses may play a critical role in initiating and/or maintaining the disease process *(5, 6)*. The association with potential T and B cell antigens, specific HLA-DR alleles, V-β TCR usage, and rheumatoid factor (RF) sequences with human disease and animal models of destructive joint disease point to such a role for T and B cells

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[4] Nonstandard abbreviations: RA, rheumatoid arthritis; TNF, tumor necrosis factor; RF, rheumatoid factor; GPI, glucose-6-phosphate isomerase; ANA, antinuclear antibody; GE, genome equivalent; MTX, methotrexate; HCL, hydroxychloroquine; SSA, sulfasalazine.

in the pathogenesis of RA. The impact of T and B cells is reflected in the improvement of disease activity after CD28 costimulatory molecule inhibition by abatacept *(7)* and B cell depletion by targeting CD20 with rituximab *(8)*.

The role of B cells and antibody in the initiation of RA has been highlighted by the finding that antibodies directed against glucose-6-phosphate isomerase (GPI), a ubiquitous enzyme in tissues, are sufficient to induce erosive arthritis in the KRN mouse. Anti-GPI antibodies bind to the antigen in tissues, including the surface of cartilage, and initiate a mast cell–dependent inflammation *(9)*. In the affected mice, mast cells are located at the invasive front of the pannus *(9)*, which parallels the histopathology of patients with RA *(10)*. Although these findings indicate that this antigen-antibody system could underlie the pathology of erosive joint inflammation in RA, there have been conflicting reports on the presence of anti-GPI antibodies in patients *(11, 12)*. Antibodies against cyclic citrullinated peptide and their target fillagrin, a component of connective tissue in the skin and cartilage, have also been linked to the pathogenesis of human RA, conceivably playing a role similar to that of anti-GPI in the KRN mouse *(13)*. In this mouse, the development of arthritis depends not only on anti-GPI antibodies, but also on the presence of mast cells; neutrophils; the complement components B, C3, and C5; and the C5a receptor *(14)*.

Antinuclear antibodies (ANAs) occur in approximately 30% of patients with RA, as determined by nuclear immunofluorescence *(15)*. Because complexes of anti-DNA antibodies with microbial or self DNA are implicated in the activation of B cells by Toll-like receptors and surface IgM RF *(16)*, it is tempting to hypothesize that anti-DNA antibodies and DNA represent such an antibody-antigen system in vivo. The present study tested this hypothesis.

Materials and Methods

STUDY PARTICIPANTS

Pilot measurements were performed on archived serum samples from healthy controls and patients with RA. The samples had been stored at −20 °C in the serum bank of the Rheumatology Department, University Hospital Basel. After this exploratory phase, cohorts of 54 patients with RA and 44 healthy controls were recruited. The criteria applied for the diagnosis of RA cases were those of the American College of Rheumatology *(17)*. The median age of the RA patients was 50 years (range 19–85 years), and the median age of the healthy controls was 45 years (range 23–80 years). The recruited patients gave written consent according to a protocol approved by the ethics review board of the Canton of Basel. Control samples were obtained either from healthy volunteers (laboratory staff) or from blood donors at the Swiss Red Cross Blood Service, Basel.

SAMPLE PREPARATION

We obtained blood samples (approximately 7 mL) by venipuncture into untreated and EDTA-coated glass tubes (Sarstedt). The blood samples were brought to the laboratory immediately for the preparation of plasma from the EDTA-coated tubes, as described *(18)*. In brief, the whole blood samples were centrifuged at 5000*g* for 10 min at 4 °C, and the plasma was transferred into microcentrifuge tubes under sterile conditions and centrifuged again at 16 000*g* for an additional 10 min. The cleared supernatant was then carefully harvested and stored at −70 °C. Serum tubes were allowed to coagulate for 55–65 min, and the serum was harvested using the above procedure.

REAL-TIME PCR ANALYSIS

We extracted DNA from 400 μL plasma or serum by use of commercial column technology (Roche High Pure Template DNA Purification Kit). We determined DNA concentrations by use of real-time PCR (TaqMan®) measurement of copies of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)[5] gene on a Perkin-Elmer Applied Biosystems 7700 Sequence Detector as described *(19)*. The detection limit of the assay was 50 copies/mL of plasma or serum.

IMMUNOGLOBULIN ADSORPTION AND ELUTION OF BOUND DNA

After 3 washes with PBS (9 g/L NaCl, 0.775 g/L Na_2HPO_4, 0.165 g/L KH_2PO_4, pH 7.4), 100 μL protein G Sepharose™ 6MB (Amersham Biosciences) was incubated with 400 μL plasma at 4 °C for 4 h. We centrifuged the plasma/protein G Sepharose mixture at 1200*g* for 3 min and carefully transferred the supernatant into a new Eppendorf microcentrifuge tube.

The protein G Sepharose pellet was gently washed 3 times with PBS, and the bound antibody was released by the addition of 150 μL tissue lysis buffer (Roche). We extracted cell-free DNA in each fraction (supernatant and antibody bound) using the Roche High Pure Template DNA Purification Kit as described above and eluted it in 40 μL elution buffer, of which 2 μL was used for the *GAPDH*-specific real-time PCR assay.

STATISTICAL ANALYSIS AND FIGURES

Statistical analysis was performed with SPSS 12.0 for Windows. We used the Mann–Whitney test for nonparametric data sets and the *t*-test for gaussian-distributed data. Statistical significance was assumed at $P < 0.05$.

[5] Human gene: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Results

SERUM AND PLASMA CELL-FREE DNA CONCENTRATIONS IN RA

In our initial exploratory examination, we analyzed 28 RA and 28 control archived serum samples. Cell-free DNA was significantly higher in the RA group than in the control group [median 69 000 genome equivalents (GEs)/mL plasma vs 37 000 GE/mL plasma; $P = 0.009$] (Fig. 1). Because the DNA concentrations in the serum may be subject to alterations from processing and circumstances of storage, or even a phenomenon peculiar to serum and not plasma, we recruited a cohort of patients with RA to verify the finding of increased circulating cell-free DNA in RA. In this analysis, plasma and serum samples from the same individuals, 26 cases with RA and 16 healthy controls, were examined in parallel.

Cell-free DNA concentrations were significantly increased in both serum ($P = 0.004$) and plasma ($P = 0.0001$) in samples from RA cases compared with the control group (Fig. 2). In addition, the amount of cell-free DNA released by clotting in generating the serum samples was much higher in the RA cases than in the control group. This increase is reflected in a higher serum-to-plasma ratio for cell-free DNA of 12.9 in the RA group compared with 7.7 in the healthy control group.

EFFECT OF THERAPY ON CELL-FREE DNA CONCENTRATIONS OF PATIENTS WITH RA

Current therapies, including those using anti-TNF–based strategies, did not appear to alter cell-free DNA concentrations. Among 12 RA patients on methotrexate (MTX), 6 on MTX and hydroxychloroquine (HCL) or sulfasalazine (SSA) therapy, 7 on MTX/HCL/SSA, and 24 on anti–TNF-α therapy, no significant treatment-related differences were apparent in plasma or serum cell-free DNA concentrations (Table 1).

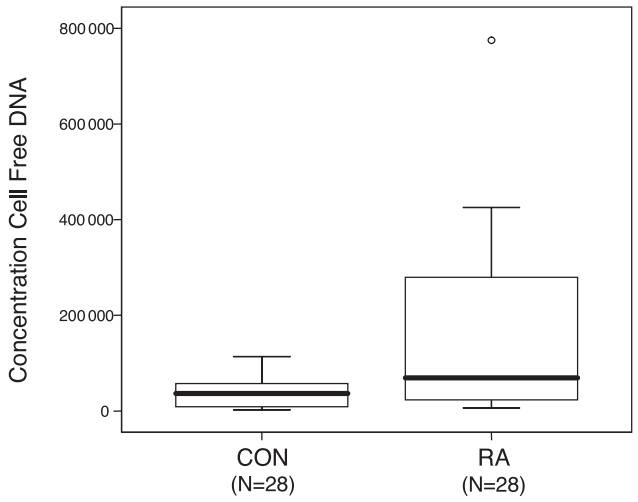

Fig. 1. Concentrations of cell-free DNA (GE/mL) from banked serum samples from healthy controls (*CON*) and RA patients (*RA*).

In the box plots, the *line* in the box indicates the median value; the limits of the box represent the 75th and 25th percentiles; and the *upper* and *lower horizontal bars* represent the 10th and 90th percentiles, respectively. The outlier is indicated by the *small circle*.

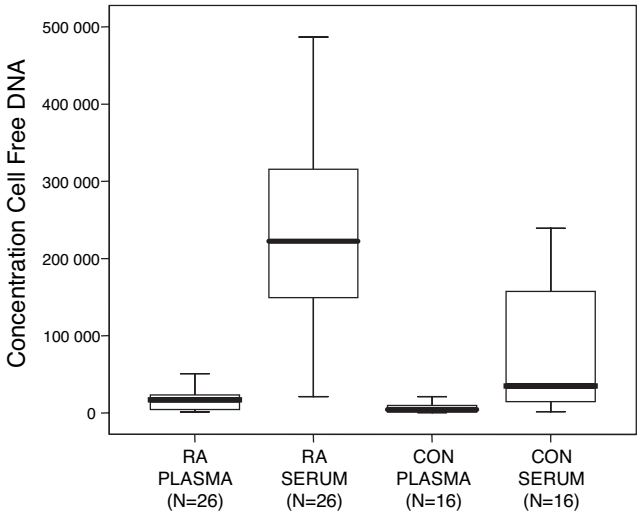

Fig. 2. Comparison of concentrations of cell-free DNA (GE/mL) in freshly obtained plasma and serum from healthy controls (*CON*) and RA patients (*RA*).

In 10 patients, blood samples were obtained before and 1 h after the infusion of infliximab, a monoclonal chimeric anti–TNF-α antibody, at a dose of 3 mg/kg body weight during a 2-h period *(3, 4)*. Of the 10 serum samples, cell-free DNA concentrations increased in serum of 5, decreased in 2, and showed no appreciable change in 3. In the plasma, cell-free DNA increased in 4, decreased in 5, and remained unchanged in 1. We noted discordant changes of values between plasma and serum in 6 cases (Table 2). In a group of 4 patients undergoing long-term therapy, no clear-cut uniform response of cell-free DNA concentrations to infliximab was discernible, with some cases showing increases and others decreases (data not shown).

CELL-FREE DNA IN RA PLASMA IS IMMUNOGLOBULIN BOUND

To determine whether cell-free DNA was associated with antibodies, plasma samples from RA patients and healthy controls were precipitated with Sepharose beads, and we measured the adsorbed and unbound fractions of cell-free DNA. In the plasma from healthy controls, the median unbound and adsorbed portions of cell-free DNA were 1000 and 100 GE/mL plasma. By contrast, in the plasma from RA cases, the median unbound and adsorbed portions were 3800 and 8000 GE/mL plasma. Hence, in the RA cases a larger percentage of cell-free DNA was associated with antibody (median 81%) than in controls without RA (9%, $P = 0.001$) (Fig. 3).

Discussion

Although the presence of cell-free DNA in plasma has been known for more than 60 years, only recently has it

Table 1. Concentrations in serum and plasma of RA patients on MTX or anti–TNF-α therapy.

	MTX	MTX + HCL or SSA	MTX + HCL + SSA	Etanercept or infliximab or adalimumab
Cell-free DNA, *GAPDH* GE/mL				
Plasma				
n	12	6	7	24
Median	17 000	18 000	17 000	17 000
Range	3000–30 000	4000–33 000	2000–80 000	2000–422 000
Serum				
n	8	3	4	24
Median	215 000	248 000	111 000	206 000
Range	149 000–487 000	21 000–280 000	22 000–189 000	21 000–487 000
C-reactive protein, mg/L				
n	12	6	7	24
Median	9.0	3.0	3.0	3.0
Range	3.0–66.0	3.0–17.0	3.0–12.0	3.0–66.0

become a focus of greater interest, largely because of the discovery of cancer-specific DNA sequences in tumor patients and fetal genetic loci in the plasma of pregnant women *(20, 21)*. Increases in total cell-free DNA have been detected in a variety of conditions, with higher concentrations of cell-free DNA attributed to increased turnover, damage, or death of cells *(22–24)*. The concentrations of cell-free DNA we measured in the plasma of healthy controls were in line with those previously reported, as were the higher concentrations in serum *(25)*. Compared with healthy controls, patients with RA showed a highly substantial increase in circulating DNA concentrations in the plasma and in the serum, which were in the high range of those reported in neoplasia *(26)* or preeclampsia *(27)*. The concentrations we found in RA patients clearly discriminated between the healthy control and RA groups in a statistically highly significant manner, indicating for the 1st time that free plasma or serum DNA may be of value as a biomarker in RA. The differences in the concentrations are all the more significant, considering the in vivo plasma half-life of DNA—in the range of minutes *(20)*.

The increased concentrations of cell-free DNA in the plasma did not vary with the administration of conventional disease-modifying therapy or between doses of TNF-α–blocking agents. Compared with concentrations before the infusion of the TNF-α–blocking monoclonal antibody infliximab, however, cell-free DNA plasma concentrations changed markedly in 7 of 10 patients. This finding is compatible with a TNF-α–related mechanism underlying DNA release. Further studies on the kinetics of DNA concentrations after infliximab, or other TNF-α inhibitors, will be needed to clarify whether the single data point after the infusion is representative for the change of DNA concentrations, or reflects 1 point of a rise and decline on a curve.

Compared with concentration increases in plasma, the concentrations of cell-free DNA in serum were disproportionately higher in the RA patients than in the healthy controls, indicating variability in the release of DNA due to clotting. Because the higher concentrations in serum have been shown to result from the release of DNA from nucleated blood cells while the thrombus retracts during clotting *(25, 28)*, the disproportionate increase is compat-

Table 2. Cell-free DNA concentrations in serum and plasma from RA patients before and after infliximab.[a]

	Plasma			Serum		
Patient	Preinfusion	Postinfusion	Fold difference	Preinfusion	Postinfusion	Fold difference
1	6000	9000	1.45	521 000	466 000	0.89
2	21 000	12 000	0.57	197 000	325 000	1.65
3	13 000	14 000	1.04	122 000	382 000	3.12
4	22 000	34 000	1.49	1 006 000	3 463 000	3.44
5	28 000	16 000	0.56	2 906 000	265 000	0.09
6	8000	14 000	1.74	29 000	57 000	1.95
7	4000	3000	0.67	31 000	36 000	1.14
8	2000	5000	2.85	183 000	15 000	0.08
9	18 000	14 000	0.75	59 000	48 000	0.82
10	20 000	5000	0.25	2 375 000	7 247 000	3.05

[a] Values of cell-free DNA are *GAPDH* GE/mL.

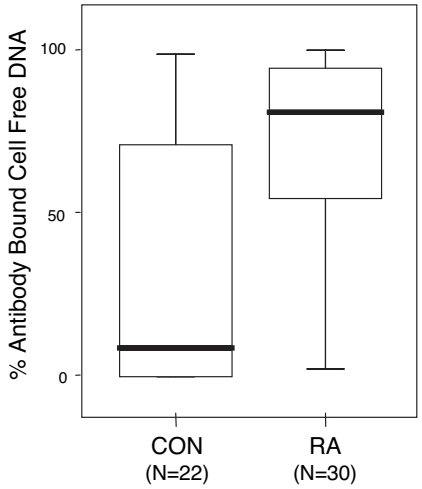

Fig. 3. Comparison of percentage of antibody-bound cell-free DNA in the plasma of healthy controls (*CON*) and RA patients (*RA*).

ible with an altered susceptibility of leukocytes from RA patients to release DNA in vitro. Although the difference in serum values between RA patients and healthy controls was highly significant, the considerable variability of the serum concentrations after the infusion of infliximab and the discordance with the plasma concentrations indicate that the determination of serum cell-free DNA concentrations may be of limited clinical value.

In addition to increased cell-free DNA, high concentrations of DNA-binding antibodies in RA plasma were detected compared with the healthy control group. Because these were adsorbed by protein G Sepharose, they must be of the IgG subclass. Typing of the involved subtypes could indicate whether the antibodies participate in complement activation. Based on the polyvalent specificity of RF for DNA and IgG *(29, 30)*, it is possible that RF plays a role in the antibody binding of cell-free DNA in the RA patients, or that antibodies of DNA specificity are masked by the high concentrations of DNA in plasma from RA patients. Such anti-DNA antibodies could be responsible for the diffuse nuclear immunofluorescence pattern present in approximately 30% of RA patients *(15)*. DNA, especially in complexes containing DNA and anti-DNA antibodies, could participate in the activation of leukocytes by Toll-like receptors and Fcγ receptors *(16)*. RFs that bind to both the Fc portion of IgG and to DNA or chromatin could also contribute to the binding of antibodies to DNA and facilitate immune complex formation *(31)*.

One possible explanation for the higher cell-free DNA concentrations in RA patients compared with healthy controls is the extrusion of DNA to form neutrophil extracellular traps by polymorphonuclear neutrophils. Neutrophil extracellular trap formation was first reported in response to endotoxin or peptidoglycan and shown to be interleukin-8 dependent *(32)*, and may also be mediated by placental trophoblast microparticles in patients with preeclampsia *(33)*. An exciting new observation is that cell-free DNA itself may be directly implicated in the etiology of RA, as suggested by a recent study on DNase II knockout mice, in which the mice developed symmetrical, erosive polyarthritis *(34)*. In these mice, in which the ability of macrophages to degrade DNA from erythroid precursors and apoptotic cells was severely reduced, macrophages produced large quantities of TNF-α and other cytokines. Further characteristics of the phenotype were the production of RF, ANA, and anti-cyclic citrullinated peptide antibodies, which are also associated with RA in humans. Given the similarity with RA, it would be highly interesting to know whether the lack of DNase II activity in this mouse model is accompanied by increased cell-free DNA concentrations in analogy to our findings.

In conclusion, the high concentrations of DNA and anti-DNA antibodies present in the plasma and serum of patients with RA represent a hitherto neglected antigen–antibody system. The presence of these antibodies not only underscores the systemic nature of the disease but may also have profound implications for our understanding of the pathogenesis of RA, especially considering the recent data from Kawane et al. *(34)*. Further studies on the origin and mechanisms of release of circulating DNA will be of interest for the underlying etiology of synovial inflammation, clinical associations, and the predictive value of alterations of circulatory DNA concentrations in therapeutic interventions. Given the presence of ANAs in systemic lupus erythematosus and other autoimmune inflammatory diseases, and their appearance under anti–TNF-α agents in patients with RA and ankylosing spondylitis *(35, 36)*, it is tempting to hypothesize that the etiology of these conditions may be linked. Further investigation of DNA and anti-DNA antibody concentrations, and the source of the DNA in these other diseases, will thus also be of considerable interest.

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