Monoclonal Anti–citrullinated Protein Antibodies Selected on Citrullinated Fibrinogen Have Distinct Targets with Different Cross Reactivity Patterns

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ABSTRACT

Objective
Anti citrullinated protein antibodies (ACPA) are thought to play a pathogenic role in rheumatoid arthritis (RA). Because of their polyclonal nature it is difficult to study characteristics of ACPA’s such as cross-reactivity or affinities. This study aimed to analyze the ACPA response at the clonal level.

Methods
Citrullinated fibrinogen specific B-cells were isolated from blood derived from an RA patient with fluorescent automated cell sorting (FACS). Antigen specificity was verified by ELISA of culture supernatant. RNA of antigen specific B-cells was isolated and VH and VL chains were cloned and subsequently expressed as IgG1 antibodies.

Results
Two human recombinant antibodies were obtained that bind to citrullinated fibrinogen peptide (cFib). Both monoclonal antibodies originated from different naive B–cells, underwent extensive somatic hyper mutation, and bind to cFib (but not to Fib) with moderate avidity. Furthermore, they showed distinct cross reactivity patterns towards other citrullinated peptides, suggesting that both antibodies have different primary targets.

Conclusion
Together these data suggest that ACPA are formed by antigen driven maturation, and that multiple citrullinated antigens are involved in activating the B cell response.
INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) such as anti-cyclic citrullinated peptide antibodies (aCCPs) are thought to play a pathogenic role in rheumatoid arthritis (RA).

ACPA are highly specific for RA, can be detected several years before the onset of disease, and are associated with disease severity.\(^1\)\(^-\)\(^3\) In animal models it was shown that transfer of monoclonal antibodies recognizing citrullinated fibrinogen or collagen into mice could exacerbate inflammatory arthritis.\(^4\) Furthermore, efficacy of B cell depletion to treat RA using rituximab strongly suggests a pathogenic role of autoantibodies.\(^5\)\(^,\)\(^6\)

ACPA, as their name implies, target citrullinated proteins. Formation of citrulline from arginine is mediated by enzymes called peptidyl arginine deiminases (PAD) and may occur during inflammatory conditions.\(^7\) PAD can essentially citrullinate all proteins, depending on the amino acids flanking the arginine. Examples of citrullinated proteins found in the inflamed synovium are fibrinogen, α-enolase and vimentin.\(^8\)\(^-\)\(^11\) So far no dominant epitope has been identified that could be the initial trigger for ACPA formation, although it is clear that the number of different citrullinated proteins recognized by ACPA is limited initially, but increases in time. This process of epitope spreading occurs prior to diagnosis of RA.\(^12\)\(^,\)\(^13\) Once RA has been diagnosed, the number of recognized epitopes remains stable.\(^12\) Another process reflecting maturation of the antibody response before diagnosis is a rise in avidity of ACPA. The avidity of ACPA rises during the same period at which the epitope spreading takes place, but avidity remains low as compared to viral recall antigens.\(^14\)\(^,\)\(^15\) This could indicate that germinal center formation ceases at a certain time point or that antigens become too abundant to drive further avidity maturation. The latter would fit with the theory of a vicious cycle of epitope spreading.\(^16\) However, the polyclonal nature of the antibody response in RA patients makes it difficult to study characteristics of ACPA’s such as cross-reactivity or affinities. In order to better define the ACPA characteristics, we aim to analyze the ACPA responses at the clonal level.
MATERIALS AND METHODS

For detailed materials and methods see supplemental material. In short, for the formation of monoclonal anti-cFib antibodies, B-cells of an RA patient fulfilling the 1987 ACR criteria for RA,\textsuperscript{17} were isolated from PBMC’s via anti-CD19 Dynabeads. The patient gave informed consent according to the declaration of Helsinki and the study was approved by the ethics committee of Reade and the Slotervaart Hospital, Amsterdam, the Netherlands. B-cells were subsequently sorted on CD27 and anti-fibrinogen expression via anti-CD27 and citrullinated fibrinogen peptide–tetramer staining respectively. Antigen specific B cells were cultured single cell for 14 days after which the specificity of the produced antibodies was verified with an anti-citrullinated fibrinogen ELISA. RNA of anti-citrullinated fibrinogen specific B cells was isolated with Trizol and subsequently cDNA synthesis and RACE PCR were performed using the Clontech SMART cDNA synthesis kit. RACE PCR products for VL and VH were cloned in pcDNA3.1 and expressed in Freestyle HEK293 cells. Nucleotide analysis was performed using the international immunogenetics information (IMGT) system.\textsuperscript{®} The antigen specificity was tested in ELISAs for citrullinated and native peptides, and Surface Plasmon Resonance (SPR) measurements were performed with a Biacore 3000 system on native and citrullinated fibrinogen.

RESULTS

Isolation and characterization of anti-citrullinated fibrinogen producing B-cells

CD27\textsuperscript{+} B-cells were enriched for citrullinated fibrinogen peptide (cFib1); 0.2% of CD27\textsuperscript{+} B-cells were labeled with cFib1. Six hundred seventy two of these cells were seeded a single cell per well and cultured for fourteen days. Afterwards, supernatants were harvested and screened for the presence of ACF antibodies by ELISA. Eight individual B-cell clones produced ACF antibodies. RNA was extracted from these clones and gamma–, kappa– or lambda–specific RACE PCR products were amplified and sequenced. Two of the antibodies were successfully expressed in HEK–293F cells by cotransfection of light and heavy chains, leading to the production of two recombinant IgG1 monoclonal antibodies: anti–cFib1.1 and anti–cFib1.2.
Citrullinated fibrinogen-specific B-cells underwent extensive somatic hypermutation.

Analyses of the VDJ genes showed that the VH of anti-cFib1.1 was formed by the usage of V4-b*02, D2-15*01 and J5*02 in combination with 63 mutations leading to 29 amino acid substitutions. The VL (lambda) consisted of V1-51*01 and J1*01 in combination with 48 mutations leading to 28 amino acid substitutions. The VH of anti-cFib1.2 was formed by combination of V1-02*02, D1-1*01 and J4*02 with 44 mutations resulting in 26 amino acid changes. The VL (kappa) of anti-cFib1.2 was based on V3-20*01 and J1*01 in combination with 21 mutations resulting in 13 amino acid substitutions. This shows that both monoclonal antibodies originated from different naive B-cells and underwent extensive somatic hyper mutation. Interestingly, both antibodies contain two N-glycosylation sites as a result of somatic hyper mutation.

Figure 1. Reactivity of monoclonal anti-citrullinated fibrinogen antibodies to different citrullinated and native peptides. The binding of anti-cFib1.1 and anti-cFib1.2 to citrullinated peptides (panel A and C respectively) and native peptides (panel B and D respectively) was tested in ELISAs. Both monoclonal antibodies were specific for citrullinated peptides without reactivity towards native peptides. They showed distinct reactivity patterns.

Recombinant anti-cFib1.1 and 1.2 react with cFib1 but not aFib1, and are cross reactive with other citrullinated peptides

The specificity of recombinant anti-cFib1.1 and anti-cFib1.2 was tested in an ELISA for anti-cFib1 peptide. Both monoclonal antibodies were specifically directed
against citrullinated cFib1 showing no reactivity with aFib1 peptide (Figure 1). Both monoclonal antibodies were also tested for reactivity towards other citrullinated peptides derived from fibrinogen, enolase and vimentin. Anti–cFib1.1 showed cross reactivity towards cFib3 and to a lesser extent towards cEno, cFib2 and cVim. Anti–cFib1.2 showed cross reactivity towards cEno and cFib2 and to a lesser extent towards cVim and cFib3 (Figure 1). This shows that both monoclonal antibodies were citrulline specific and had distinct cross reactivity patterns.

**Figure 2.** Binding of monoclonal anti–citrullinated fibrinogen antibodies to citrullinated fibrinogen (cFib) and fibrinogen (Fib). Binding to cFib and Fib of 10 µg/ml anti cFib 1.1 (A) and 1.2 (B) as measured by SPR. Dotted line represents fit ($k_a = 2.6 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_d = 1.6 \times 10^{-3}$ s$^{-1}$). C) Binding of anti–cFib 1.1 or Fab fragment thereof to cFib. D) RU at $t = 800$ s vs concentration of Fab 1.1. Solid line represents fit of a 1:1 binding model (Langmuir) resulting in a $K_d$ of 700 nM.

**Affinity of anti–cFib1.1 and 1.2.**

Binding of both antibodies to citrullinated fibrinogen was also assessed with SPR measurements. Both antibodies bind to surface–bound citrullinated fibrinogen, but not native fibrinogen (Figure 2A, B). Anti–cFib 1.1 showed a complex binding profile and therefore avidity could not be calculated. The complex binding profile can be partly explained by monovalent vs bivalent binding, but binding profiles of Fab fragments of anti–Fib 1.1 were also indicative of multiple modes of binding (Figure 2C), probably as a result of multiple citrullinated sites on the fibrinogen. An apparent affinity constant of $K_d = 700$ nM was estimated from end–point RU values.
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(Figure 2D). For anticFib1.2, an apparent avidity of $K_d = 50$ nM was obtained from the calculated association and dissociation constants (Figure 2B).

DISCUSSION

A detailed study of the ACPA antibody response in terms of specificity is severely hampered by its polyclonal nature. We developed a method to clone human monoclonal ACPA resulting in the development of two monoclonal IgG1 antibodies to citrullinated fibrinogen. We showed that these monoclonal antibodies are specific for citrullined fibrinogen peptides, but with distinct (cross-)reactivity patterns towards other citrullinated peptides derived from other proteins. We also showed that these antibodies were derived from different naive B cells that underwent extensive somatic hypermutation. The avidity of citrullinated fibrinogen binding is nevertheless moderate, indicating that citrullinated fibrinogen is not the primary antigen that drove differentiation of these B-cell clones. Notably, the VH of one of the antibodies was derived from V4-b*02, a germline gene that was previously described to be the most prevalent in a phage display library of aCCP antibodies.

We and others have previously shown with inhibition assays that cross reactivity between ACPA appears to be limited. The results of the present study indicate that ACPA can be cross reactive, but the fact that both antibodies show distinct cross reactivity patterns indicates that this reactivity is indeed restricted. Nevertheless, this limited cross reactivity of ACPA implies that large immune complexes may be formed between ACPA and their various antigens, especially if IgM–RF is also present. Larger immune complexes might have a more pronounced inflammatory effect due to complement activation and FcγR triggering. This could explain why arthralgia patients with an extended ACPA repertoire and IgM–RF have more risk of developing arthritis and supports the hypothesis that a certain threshold has to be reached in the number and titer of ACPAs before patients progress to RA.

This study has certain limitations. First, we only cloned two antibodies of one patient and thus, this study should be regarded as a proof of concept study. For a detailed analysis more antibodies of more patients should be cloned. Second, by screening B cells with an ELISA for anti-citrullinated fibrinogen, a selection bias was introduced. In future research it may be possible to screen unselected B-cells for multiple citrullinated peptides with higher sensitivity, for instance with microchip-based assays. This will also allow the cloning of antibodies to other citrullinated proteins. In the present study, we only cloned ACPA for citrullinated fibrinogen, but
it would be interesting to see the characteristics of monoclonal antibodies for other citrullinated proteins as well.

In conclusion, we cloned two monoclonal ACPAs derived of an RA patient. Our results indicate that ACPA have distinct cross reactivity patterns and are formed by antigen driven maturation.

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SUPPLEMENTARY MATERIAL: DETAILED MATERIALS AND METHODS

Antibody assays
Anti–citrullinated fibrinogen (ACF) antibodies were detected with an ELISA as previously described.\(^1\) In short, IgG depleted fibrinogen was citrullinated using rabbit skeletal muscle PAD and coated on maxisorp microtitre plates. Supernatants were incubated 1:5 for one hour at room temperature on the coated plates and ACF antibodies were detected with horseradish peroxidase conjugated mouse monoclonal antihuman IgG (HRP–IgG) and visualized with 3,3′,5,5′-tetramethylbenzidine (TMB).
Antibodies were tested for reactivity towards different citrullinated peptides as previously described.\(^1\) In short, sera were incubated on streptavidin plates coated with biotinylated, citrullinated or native peptides. Bound antibodies were detected with HRP–IgG and then visualized with TMB. Reactivity was expressed as difference in optical density (ΔOD) between citrullinated and native peptides.

Citrullinated fibrinogen peptide tetramers
Biotinylated citrullinated fibrinogen peptide NEEGFFSACitGHRPLDKK (0.5 mg/mL) was mixed with APC labeled streptavidin (Molecular Probes) (0.5 mg/mL) in PBS/0.1%BSA and incubated on a roller bank over night at 4ºC in the dark. Tetramers were purified over a bio–spin 30 column (Biorad) according to the manufacturer’s protocol.

Isolation, proliferation and identification of citrullinated fibrinogen specific single B–cells
For the isolation of B–cells, citrated blood was collected from an RA patient positive for aCCP. The study was approved by the local ethics committee. Percoll gradients (Amersham Pharmacia Biotech, Uppsala, Sweden) were used to isolate peripheral blood mononuclear cells (PBMC’s) from citrated blood. B–cells were isolated using anti–CD19 Dynabeads and DETACHaBEAD (Invitrogen, Paisley, UK), according to the manufacturers instructions. Isolated B–cells were sorted for antigen–specificity by FACS sorting (BD FACSaria II) using anti–CD19–PerCP–Cy5 (BD Biosciences, San Jose, USA), anti–CD27–PE (BD Bioscience) and APC labeled fibrinogen peptide tetramers. Cells were seeded 1 cell per well in 96 well flat bottom plates and cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Lonza, Basel, Switzerland) containing 10% FCS (Bodinco, Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 50 μM β–
mercaptoethanol (Sigma–Aldrich), 20 µg/ml human IgG depleted apo–transferrin (Sigma–Aldrich) 1 ng/ml II–1β, 50 U/ml II–2, 0.3 ng/ml TNFα, 0.5 µg/ml R848 (Alexis, Lausan, Switzerland) in the presence of 1.10^5 irradiated (50 Gy), CD40L–expressing EL4–B5 cells. After 14 days, supernatants were tested for ACF antibody production in an ELISA as described above.

Production of recombinant human antibodies
RNA was isolated from antigen specific B–cells with Trizol (Peqlab, Erlangen, Germany) according the manufacturer’s protocol. cDNA synthesis and RACE PCR were performed using the Clontech SMART cDNA synthesis kit (Clontech, Mountain view, CA, USA). RACE PCR products for VL and VH were cloned into pGEM–t easy (Promgea, Madison, WI, USA) and sequenced with Big Dye Terminator (Aplied Biosystems, Foster City, USA) according to the instructions provided by the manufacturers. VL and VH sequences followed by the constant domains of the human Kappa or Lambda and human IgG1 were ordered at Mr Gene (mr Gene GmbH, Regensburg, Germany) and cloned in pcDNA3.1 (Invitrogen) expression vectors. Expression vectors were used for transient transfection of HEK293F cells with 293fectin and OptiMEM (Invitrogen), using the Freestyle HEK293F expression system (Invitrogen) according to the manufacturer’s instructions.

SPR
SPR measurements were performed with a Biacore 3000 system (Biacore AB, Breda, The Netherlands) at 25 °C. Fibrinogen, citrullinated fibrinogen, and human serum albumin (reference channel) were bound covalently to CM5 sensor chips at a concentration of 5 µg/mL in 10 mM sodium acetate pH 4, using N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Binding of anti–cFib antibodies (or Fab fragments, obtained by papain digestion) in 10 mM HEPES, pH 7.4, containing 3.4 mM EDTA, 0.15 mM NaCl, and 0.005% Tween 20 was measured at a flow rate of 20 µL/min. After each run, bound material was removed in a regeneration step by injecting 5 µL of 0.1 M H_3PO_4.

Nucleotide sequence analysis
Nucleotide analysis was performed using the international immunogenetics information (IMGT) system®
REFERENCES


