Recombinant Human Monoclonal Autoantibodies Specific for Citrulline-Containing Peptides from Phage Display Libraries Derived from Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To isolate and characterize monoclonal autoantibodies (Mab) directed to citrullinated antigens from patients with rheumatoid arthritis (RA).

Methods. Using lymphocytes from bone marrow or peripheral blood from RA patients, we constructed antibody fragment libraries representing the antibody repertoire of these individuals. Antibody fragments recognizing a citrulline-containing peptide were selected from these patient libraries. Individual antibody clones were analyzed for germline gene usage and reactivity toward citrullinated (auto)-antigens.

Results. Sequence analysis of the cDNA encoding the 21 distinct antibody fragments that were obtained revealed a restricted germline gene usage. Individual antibody clones were positive in both antiperinuclear factor (APF) and antikeratin antibody (AKA) tests, stained citrullinated filaggrin and fibrinogen on Western blots, and reacted with subsets of citrulline-containing peptides in ELISA, but not with noncitrullinated peptides.

Conclusion. Our report describes the first recombinant human Mab fragments reactive with citrulline-containing peptides. The restricted germline gene usage of these antibodies, and the fact that the VH alleles used are not present in all individuals, may indicate the existence of a genetic predisposition for the development of anticitrulline antibodies in individuals with these germline alleles. The selected antibody clones may facilitate studies on the role of these autoantibodies and their target antigens in the development of RA. (J Rheumatol 2003;30:1696–711)

Key Indexing Terms:
RECOMBINANT ANTIBODY FRAGMENTS CITRULLINE-CONTAINING PEPTIDE
ANTICITRULLINE SINGLE CHAIN VARIABLE FRAGMENTS RHEUMATOID ARTHRITIS

The etiology of autoimmune diseases such as rheumatoid arthritis (RA) is still unknown. Autoimmune diseases are characterized by the occurrence of an immune reaction towards self-antigens, and often a specific correlation between the type of autoantibody target and the type of rheumatic disease can be found. Consequently, such autoantibodies can be very useful for diagnosis and prognosis of rheumatic diseases[1]. In rheumatoid arthritis (RA), serological support for the diagnosis is not well established and is mainly based on the presence of rheumatoid factors (RF), which are not very specific for RA[2].

Recently, a novel and very specific autoantibody system in RA, based on the detection of citrullinated residues in proteins, has been described[3,4]. Citrullination (or deimination) is catalyzed by the enzyme peptidylarginine deiminase and involves the deimination of arginine in a peptide context[2]. It was found that such antibodies (here referred to as anti-CCP: anticitrulline-containing peptide) not only are very specific for RA (close to 98%), but they are also present very early in the disease[4,5], and high titers of these antibodies are indicative for erosive disease[5,6]. Anti-CCP antibodies are comparable to the previously described antiperinuclear factor (APF) and the so-called antikeratin antibodies (AKA) because in these systems as well a citrullinated substrate (filaggrin) is the antigen[7]. The actual citrullinated autoantigen in synovial tissue is not known (filaggrin is not present in the synovium), although a recent study has shown that the α- and ß-fibrin(ogen) chains are citrullinated in RA synovia[8].

To investigate anti-CCP autoantibodies present in patients with RA, we applied phage display technology to

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select a panel of anti-CCP autoantibodies from RA patient derived phage display libraries. Twenty-one single-chain variable fragments (scFv) were selected. Their specificity and gene usage were analyzed in detail.

MATERIALS AND METHODS

Patient material. Sera. RA patient sera and sera from healthy volunteers were collected at the Department of Rheumatology of University Medical Centre St. Radboud. Sera were stored at –80°C until use. Patients were diagnosed according to the criteria of the American College of Rheumatology[9]. Sera were tested in an ELISA for anti-CCP reactivity as described below. Informed consent was obtained from all participants according to the medical ethical regulations.

Bone marrow, peripheral blood, and apheresis material. Bone marrow, peripheral blood, and apheresis samples were collected from selected donors at the Department of Rheumatology. Donors were selected from a group of patients with RA based upon the presence of anti-CCP antibodies in their sera.

Immunoblotting, preparation of cell extracts, and purification of filaggrin. Citrullinated filaggrin was extracted from human epidermis as described[3,10]. Fibrinogen (fraction I from human plasma; Sigma-Aldrich Chemie, Steinheim, Germany) was enzymatically deiminated *in vitro*, essentially as described[8]. Citrullination was monitored as described[11,12]. Total cell extracts of HeLa and HEp-2 cells were prepared as described[13]. Filaggrin (0.5 µg), citrullinated and noncitrullinated fibrinogen (0.5 µg), and cell extracts were run on a 10% SDS-PAGE gels (BioRad sodium dodecyl sulfate-polyacrylamide minigel system; BioRad, Hercules, CA, USA) and were electroblotted to nitrocellulose.

Blot strips were blocked with 5% nonfat dry milk (Marvel) in TBST (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% Tween-20) and incubated with human sera diluted 1:50 in the same buffer, with bacterial culture supernatants containing scFv/phages, or, after chemical modification of the citrulline residues[12], with anti-(chemically modified)-citrulline antibodies (a kind gift of Dr. T. Senshu, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) for 2 h. After washing, the strips were incubated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated secondary antibodies (anti-human IgG or anti-rabbit Ig, 1:1000; Dako, Glostrup, Denmark), anti-VSV (vesicular stomatitis virus)-G tag antibody (1:1000, derived from hybridoma cell line P5D4), or anti-M13 (1:5000; Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature. Unlabeled secondary antibodies were detected with tertiary HRP or AP labeled antibodies (anti-mouse Ig, 1:2000; Dako). For detection the strips were developed using NBT/BCIP or chemiluminescence.

Peptide synthesis. Peptides cfc1-cyc (HQCQESTXGRSRGRCGRSGS; X = citrulline residue) and cf0-cyc (HQCQESTRGRSRGRCGRSGS) have been described[3]. Cfc1-cyc peptide was selected because of its reactivity with a large number of RA sera. Linear and cyclic versions of citrullinated (cfc1 and cfc1-cyc, respectively), noncitrullinated (cf0 and cf0-cyc, respectively), and biotinylated and nonbiotinylated peptides, as well as a set of linear citrullinated and noncitrullinated peptides related to cfc1-cyc were synthesized and purified as described[3]. The purity of the peptides was at least 98%. Biotinylated (biot) peptides contained N-terminal biotin. All peptides contained a C-terminal amide group.

Peptide ELISA. The ELISA in which peptides (1 µg peptide/well) were used was performed on DNA-bind assay plates (Costar, Cambridge, MA, USA) essentially as described[3,4]. ScFv or phages were diluted (2- to 10-fold) in 5% Marvel in phosphate buffered saline (PBS). Anti-VSV (P5D4; 1:5000 in 5% Marvel PBST) or anti-VSV HRP conjugate (1:1000 in 5% Marvel PBST) was added to wells incubated with scFv. Anti-M13 antibodies or HRP conjugated anti-M13 antibodies (Amersham) diluted 1:5000 in 5% Marvel PBST were added to the wells incubated with phages. After incubation (1.5 h at room temperature) plates were washed (6 times with PBST) and bound HRP conjugated antibodies were detected with K-Blue substrate (Neogen, Lexington, KY, USA).

Introduction of VSV-G, VSV-G-His6, and Cκ-domain tags in phage display vectors. The 11 amino acid vesicular stomatitis virus derived VSV-G tag (YTDIEMNRLGK)[14,15] was introduced into the pHENIX vector[16] by polymerase chain reaction (PCR) using oligos VSV-NotI 5'AAGGAAGAATGCGGCCGCATATACAGACATAGAGATGAACCGACTTGGAAAGGGGGCCGCATAGACTGTTGAAAGTTGTTTA3' and pHENBACK 5'GTTGAGGCAGGTCAGACGATT3', resulting in the pHENIXVSV vector. A VSV-G + His6 tag fragment was PCR amplified from the pHENIXVSV vector using oligos LMB3 5'TCACACAGGAAACAGCTATGAC3' and VSVHis-EcoRI 5'CGGAATTCTTATTAATGGTGATGATGGTGATGTGCGGCCCCTTTCCAAGTCGGTTCATCTCTATGTCTG3' and introduced into the pUC119MycHis6Sfi/Not vector[17], resulting in the pUC119VSVHis6Sfi/Not vector. The mouse Cκ-domain was used because it often introduces an increased stability to the antibody without altering its antigen recognition capacities. It was amplified from cDNA derived from mouse spleen lymphocytes using oligos CκmForNot-I 5'GAGTCATTCTCGACTTGCGGCCGCGGCTGATGCTGCACCAACTGTATC3' and CκmBackBgl-II 5'GAAGATCTCGCGGCTGCACACTCATTCCTGTTGAAGCTC3'. PCR fragments were digested with Not-I and Bgl-II and cloned in a Not-I Bgl-II digested pUFosVH6 vector, resulting in pUCmκ-VH6. The pUFosVH6 vector was created by first introducing the Fos dimerization domain[18] as a *Not*I fragment in the pUC119VSVHis6Sfi/Not vector. Subsequently, the Fos fragment was amplified from this vector using oligos LMB3 and Fos 5'ATAAGATCTGCAACCACCGTGTGC3'. Simultaneously, a VSV-G-His6 fragment was amplified from the same vector using oligos VSVBglII 5'GGTGGTTGCAGATCTTATACAGACATAGAGATGAAC3' and M13Forward 5'CGCCAGGGTTTTCCCAGTCACGAC3'. In a subsequent overlap PCR, both products (Fos and VSV-G-His6) were amplified with oligos LMB3 and M13Forward, digested with *Not*I and *Eco*RI, and ligated in a *Not*I and *Eco*RI digested pUC119MycHis6Sfi/Not vector.

cDNA synthesis, PCR amplification, and cloning of scFv heavy and light chain genes. RNA was extracted from bone marrow samples from RA patients (1.5×10^7 cells from Patient b and 5×10^6 cells from Patient c) or from apheresis of a patient (2.5×10^8 cells; Patient a). cDNA was synthesized according to Finnern, *et al*, except for the first-strand synthesis, in which oligo-dT primers were used. Libraries were generated using PCR reactions essentially as described[19]. ScFv genes were assembled in a 2-step procedure, in which light and subsequently heavy chains were cloned sequentially into the phagemid vector pHENIXVSV. Libraries were tested for their maximum number of phagemid clones, for their full-length scFv cDNA content by PCR analysis, and fingerprint analysis performed by Bst-NI restriction digest[20]. For an overview of patient library characteristics see Table 1.

Selection of peptide binders: solid phase selection using nonbiotinylated peptide. Selections were performed initially using nonbiotinylated peptide covalently coupled to 96-well DNA-bind assay plates (Costar). The citrullinated peptide was coupled at 10 µg/well in PBS/0.5 M NaCl adjusted to pH 9.0 for 1 h. PBS used in subsequent steps was pH 7.3. For the solid phase pannings we used the selection and reamplification protocol described by Marks, *et al*[21]. To 12 peptide coated wells, 0.1 ml/well phage antibodies (10^{13} TU in PBS/5% Marvel) from the individual libraries were added. After the selection, washing, and elution steps[21], phage rescue was performed by infection of *E. coli* TG1 with the selected phages. Selection rounds were repeated 4 times.

Soluble phase selection using biotinylated peptides. In a second round of selections all patient libraries were pooled and selections were performed with biotinylated peptide in solution. For each selection, 200 µl streptavidin beads (Dynal) were blocked with 4% MPBS for 2 h at room temperature on a rotating platform. One milliliter of phage library (> 10^{12} pfu/ml) was preincubated with 100 µl streptavidin beads to preabsorb any streptavidin binders. Subsequently, 1 µg biotinylated peptide was mixed with the "library mix" supernatant from the preselection and incubated for 60 min

Table 1. Patient phage display scFv libraries. Library size = the maximum number of different antibody clones in the library. Percentage full-length = percentage of phagemid clones with full-length scFv cDNA.

RA Patient Library	Size	Starting Material	Percentage Full-Length
LIgG a	VH/VLκ 4 x 10^7	Apheresis	96
	VH/VLλ 2 x 10^7		83
LIgG b	VH/VLκ 2 x 10^7	Bone marrow	92
	VH/VLλ 1 x 10^8		75
LIgG c	VH/VLκ 1 x 10^8	Bone marrow	88
	VH/VLλ 5 x 10^7		75

on a rotating platform. The peptide phage mix was added directly to 100 µl Dynabeads and incubated for 15 min at room temperature on a rotator. Tubes were placed in a magnetic rack for 1 min, then the supernatant was removed carefully. Beads were washed 5 times with 4% MPBST, transferred to an eppendorf tube, and washed again 5 times with PBST. The beads were transferred again and washed twice with PBS. Phages were eluted sequentially with 100 mM triethylamine (10 and 20 min with 0.5 ml). Eluates were neutralized with a total of 0.5 ml 1 M Tris-HCl, pH 7.4, directly after elution. For the soluble phase pannings, the phage rescue and reamplification protocol described by Barbas, *et al*[22] was used. Selection rounds were repeated 4 times.

Analysis of selected phage clones. Phage ELISA. All selected phage pools were analyzed by ELISA for binding to the peptide they were selected against. From the second round onwards, 96 individual phages per selection round were analyzed by ELISA to determine whether they bound to the respective antigenic peptides.

PCR and fingerprint analysis. PCR analysis and fingerprinting (digestion with Bst-NI of PCR amplified fragments[20]) were performed to check whether selected clones contained full-length scFv coding sequences and to analyze whether individual bacterial clones contained different scFv coding regions.

Soluble scFv expression and specificity ELISA. For soluble scFv expression, easy detection, and purification, binding clones were recloned into a pUC119VSVHis6Sfi/Not or in the pUCmκ-VH6 vector, respectively. For determining the specificity of the selected clones, various antigens (0.3 µg/well) were coated (in 50 mM $NaHCO_3$, pH 9.6, o/n at room temperature) to a 96-well Maxisorb (Nunc) ELISA plate. Soluble scFv from the bacterial culture supernatant was diluted 1:1 in MPBST. As control antibodies for testing proper coating of the antigens, various recombinant scFv obtained from nonrelated selections were used (Raats, unpublished data)[23].

Purification of scFv. Bacteria containing the recloned scFv constructs were grown in 0.5 l cultures and scFv expression was induced by addition of IPTG as described[24]. ScFv were purified from periplasmic fractions by immobilized metal chelate affinity chromatography, and dialyzed o/n against PBS, pH 8.0, essentially as described[23].

Sequencing of scFv coding regions. ScFv coding regions were sequenced using oligonucleotidess LMB3, Forlinkseq 5'GCCACCTCCGCCTGAACC3', Revlinkseq 5'GGTTCAGGCGGAGGTGGC3', and FdSeq1 5'GAATTTTCTGTATGAGG3'.

Indirect immunofluorescence. Detection of APF antibodies by immunofluorescence on buccal mucosa cells was performed as described[25]. The serum dilution was 1:10. ScFv or scFv-Cmκ fragments were obtained as periplasmic fractions, Ni-agarose purified, and dialyzed against PBS (o/n) and used at a concentration of 0.1 to 1.0 mg/ml for IFA. Buccal mucosa cells from a single donor were used throughout this study.

Detection of AKA antibodies was performed on cryosections of rat esophagus prepared as described[26].

For testing cross-reactivity of recombinant antibodies with cellular components, HeLa cells (American Type Culture Collection CCL-2) and HEp-2 cells (ATCC CCL-23) were grown on coverslips, washed twice with PBS and once with distilled water, fixed in methanol/acetone for 10 min at –20°C, dried, rehydrated in PBS for 5 min, and stained with scFv (0.1 to 1.0 mg/ml) for 1.5 h, followed by 3 washes with PBS and detection with mouse anti-VSV-G and rabbit anti-mouse FITC-labelled antibodies or with anti-mouse Ig antibodies labelled with FITC (Dako).

Competition studies. Competition for binding to peptide cfc1-cyc conjugated to bovine serum albumin (BSA; glutaraldehyde conjugation as described[27]) with a panel of recombinant anti-CCP antibodies, RA patient sera, and noncompeting control sera was studied. Antibodies that were available as scFv-Cmκ fragments (more stable) were used in that format (indicated by "*" in Figure 4); the others were used in the scFv format. ScFv-Cmκ or scFv fragments of the various recombinant antibodies were premixed with a serial dilution of the competing patient serum (serum b, c, or a combination of 8 RA patient sera, m). Equal amounts of noncompeting control serum were used as a negative control. Recombinant antibodies were used in a dilution resulting in an optical density (OD) 450 of 2/3 of the maximum OD in the linear range of the binding curve of the recombinant antibodies as determined by endpoint titration. Bound recombinant antibodies were detected with HRP-linked rabbit anti-mouse (Dako) (for the Cmκ clones only), or with anti-VSV-G followed by HRP-linked rabbit anti-mouse (Dako) for all other recombinant clones. Percentage competition was plotted. The OD 450 signal of the blank (no recombinant antibody added) was defined as 100% inhibition and the signal with noncompeting control serum added to the recombinant antibody incubation was defined as 0% inhibition. After serum/scFv incubation for 3 h, plates were treated as described in the ELISA section.

RESULTS

Phage display. Elucidation of the genetic repertoire of genes encoding the variable heavy and light chain domains of human antibodies[28-30] allowed the design of oligonucleotides that enable PCR amplification of the complete antibody encoding cDNA repertoire from an individual. The cloning of this repertoire into phagemid or phage vectors enables the display of such antibody repertoires on the surface of filamentous phages. Using various panning methods, individual antibody specificities can subsequently be selected and amplified from the phage-displayed antibody repertoires (antibody phage display libraries). Because in phage display systems the genotype (antibody variable region genes) is physically linked to the phenotype (antibody binding specificity), a cDNA (encoding the antibody with the desired binding specificity) and its corresponding antibody fragment are selected simultaneously. Such variable antibody domain-encoding cDNA can be analyzed in detail (e.g., germline gene usage, mutation frequency) and can be cloned into various expression systems for optimal use of the encoded antibody fragment in different assays and applications. For an extensive overview of phage display technology, see Hoogenboom, *et al*[31].

To study the autoantibody repertoire of patients with RA, we constructed antibody fragment libraries from the B cell repertoire of 3 individual patients with RA (libraries a, b, and c), as described above. The complexity (maximum number of different phagemid clones) of the scFv phage display libraries varied between 0.6×10^8 and 1.5×10^8 (Table 1).

Selection of anti-CCP clones. Individual phage libraries were subjected to 4 rounds of affinity selection on citrullinated cyclic peptide cfc1-cyc. Two selection methods were applied: (1) solid phase selection using the cfc1-cyc peptide bound to DNA-bind plates, and (2) soluble phase selection using biotinylated cfc1-cyc peptide and streptavidin coated beads. Method 1 was performed with the individual libraries LIgGa, b, and c. Method 2 was performed with a combination of the 3 libraries.

With selection method 1 a total of 81 reactive scFv were obtained. Fingerprint analyses showed that these clones could be subdivided into 25 groups (one from LIgGa, 11 from LIgGb, and 13 from LIgGc). However, only one single clone derived from library LIgGa (clone Ra3) proved to be truly specific for the citrullinated peptide and showed no cross-reaction with the noncitrullinated counterpart (cf0-cyc). All other selected clones showed weak cross-reactivity with cf0-cyc and, in general, displayed inefficient binding capacities in ELISA. Since our objective was to study anti-CCP antibody clones, further analyses were performed only with clone Ra3 derived from library LIgGa.

With selection method 2 we obtained 198 reactive scFv clones. Although subdividing the reactive clones via *Bst*-NI restriction pattern analysis (fingerprinting) of their respective cDNA has slight potential for introducing bias, we used this approach to subdivide the selected clones into 27 fingerprint groups. From these 27 different scFv families only 20 showed sufficient binding and specificity for citrullinated peptides in ELISA to enable further analysis.

Specificity of scFv. To investigate the specificities of the selected scFv in more detail, ELISA were performed using a panel of (auto)antigens, e.g., Jo-1, CENP-B, Ro52, hTopoI, U1A, La, Annexin XI (for an overview of autoantigens see[1]), HspB3[32], and BSA. In total, 21 individual clones were selected based on their fingerprint and soluble scFv expression concentrations (from each fingerprint group the clone that showed the highest expression level was chosen). From these clones, D7-2 and F12-2 showed slight cross-reactivity with the human Ro52 protein, and clone F12-2 also showed cross-reactivity with the human Annexin XI protein. No other clone revealed detectable cross-reactivity in this assay (Figure 1).

To investigate the epitopes targeted by the scFv, their reactivities were studied in ELISA with several peptides covering different regions of the citrullinated peptide that was used for the selections and with nonrelated peptides containing citrulline residues and their noncitrulline-containing counterparts. All clones reacted exclusively with the cfc1-cyc peptide used for the selections. Replacement of the citrulline residue in the cfc1 peptide by Arg, Ala, Glu, or Gln completely abrogated the reactivity with all clones. Similar results were obtained with phages and soluble scFv, although the signals obtained with the latter were somewhat lower. Based on their reactivities with citrullinated peptides the antibody clones were subdivided into 3 reactivity groups. For an overview see Table 2. Of special interest is clone Ra3, which reacted (although in varying degrees) with almost all peptides that contained a citrulline residue. Other clones from reactivity group 1 (A2-2 and F12-2) seemed to be slightly more selective for the peptide context of the citrulline residue (e.g., loss of reactivity with peptide cf18-31).

Within reactivity group 2 we observed an increase in the requirement for a particular peptide context. In contrast to the group 1 antibody fragments, none of the group 2 antibody fragments reacted with the cfc4 and cfc5 peptides. The clones from group 2b also lost reactivity with peptide cf18-34-3.

Finally, clones of group 3 also lost reactivity with peptide cfc3 and recognized only a subset of the 19 amino acid-long cfc peptides with a citrulline residue (cfc6 to cfc9). Patient sera a, b, and c displayed a peptide recognition pattern similar to that observed for the recombinant antibody clones. These analyses showed that within the citrullinated peptides both the number and the location of the citrulline residues determine (at least in part) the level of reactivity with the recombinant antibodies.

AKA and APF test with selected antibody fragments. To determine the staining patterns of the recombinant antibodies in 2 tests often used for RA autoantibodies, i.e., the APF (human buccal mucosa cells) and AKA (rat esophagus sections) immunofluorescence tests, scFv or scFv-Cmκ fragments (containing the mouse light chain constant Cκ domain for increased stability) were used. In the APF and AKA tests the reactivity of the recombinant antibodies was compared with that of the respective parent serum. Although the intensity of immunofluorescent staining differed between the various recombinant antibodies in both the APF and AKA tests, they displayed identical staining patterns compared to patient sera (Figure 2; Table 3, immunofluorescence analyses for APF, AKA). It is notable that compared to RA patient sera the recombinant antibody staining patterns displayed much lower background staining in both these tests.

Western blotting. The reactivity of the recombinant antibodies with citrullinated proteins such as filaggrin and citrullinated fibrin(ogen) subunits was investigated. These proteins have been shown to be reactive with RA patient sera[3,8]. We used phages, scFv or Cκm-scFv fragments, RA patient sera, and Senshu antibodies[12] to stain Western blots containing filaggrin and noncitrullinated and *in vitro* citrul-

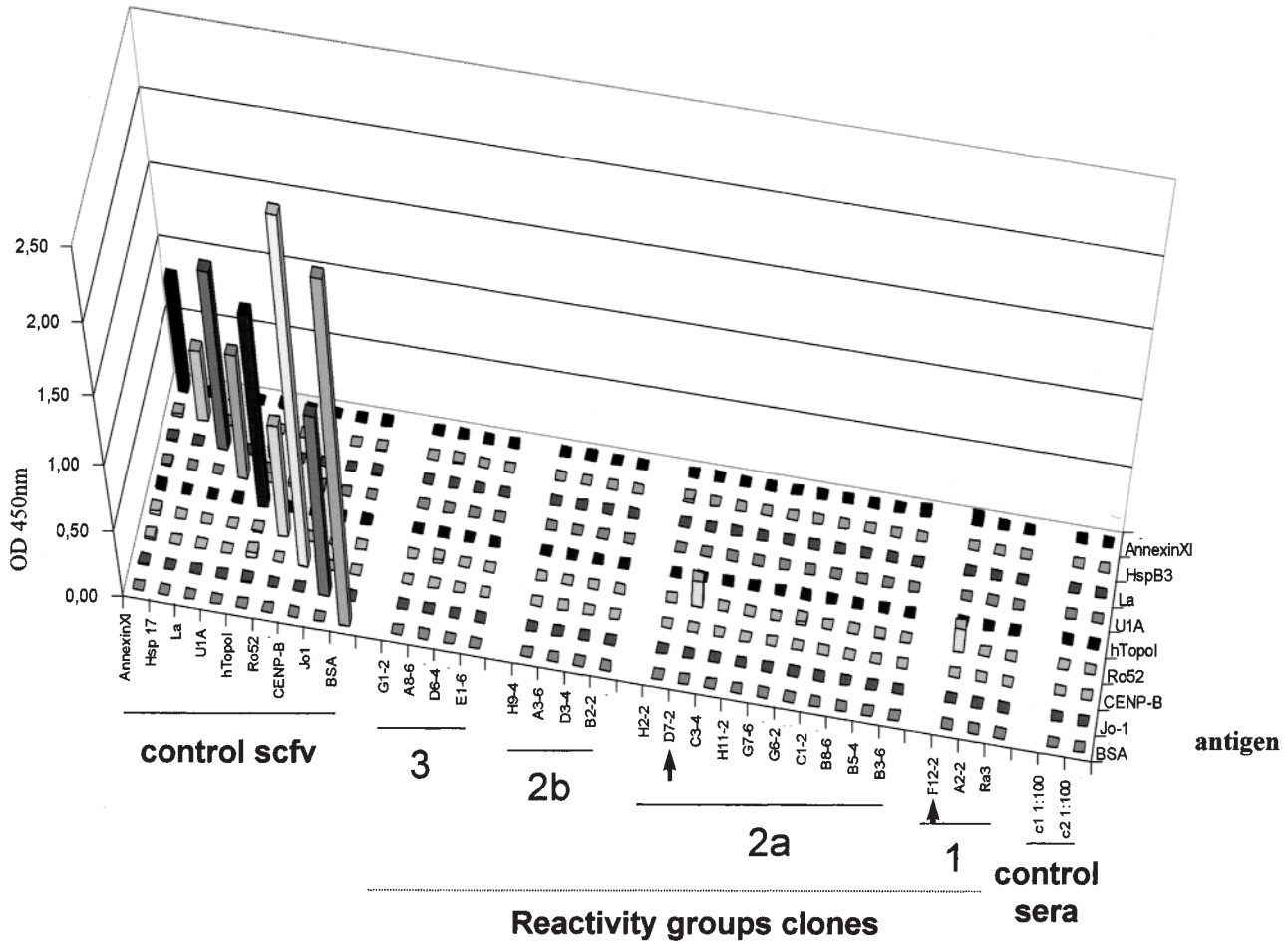

Figure 1. Specificity of recombinant anti-CCP antibodies measured by ELISA. Only 2 of the 21 antibody clones (arrows) showed low cross-reactivity: D7-2 and F12-2 with human Ro52, and F12-2 with human Annexin XI antigen.

linated fibrinogen. These Western blot experiments revealed that all scFv clones except D7-2 and G1-2 reacted with the filaggrin protein (data not shown). All clones tested were reactive with the Aα chain of citrullinated fibrinogen except for clone E1-6, which reacted only with a high molecular mass band ~200 kDa that appears after *in vitro* citrullination of human fibrinogen[8] (Figure 3b, Table 3). Some clones also displayed reactivity with the Bß chain and/or the high molecular mass ~200 kDa band (Figure 3). The reactivity of these scFv with the Bß chain might correlate with their reactivity with certain peptides (i.e., cfc4 and cfc5). This may be related to the context of the citrulline residue(s) in these (poly)peptides. However, since the reactivity of most scFv with the Bß fibrinogen chain on Western blot and the synthetic peptides in ELISA is generally weak, no definite conclusions can be drawn. Note that some clones (D7-2, G1-2, and B2-2) appear to react exclusively with the Aα chain. The reactivity of recombinant antibodies with citrullinated fibrinogen is similar to the reactivities of patient sera with this antigen (Figure 3a)[8]. Serum c displayed a strong and serum b a weak reactivity with the Aα and Bß chains of citrullinated fibrinogen, whereas serum a reacted only very weakly with the Aα band, and in addition reacted with a band migrating slightly faster than the Aα band reactive with serum c and b. All antibodies tested showed no or negligible reactivity with noncitrullinated fibrinogen (compare panels "citrullinated fibrinogen" and "fibrinogen" in Figure 3).

No reactivity of the recombinant antibodies with components of HeLa or HEp-2 cells was observed in immunofluorescence tests with HeLa or HEp-2 cells or on Western blots containing HeLa or HEp-2 cell extracts (data not shown).

Competition assays with patient sera. To analyze whether the recombinant antibodies react with similar epitopes as antibodies present in RA patient sera, we performed competition assays of scFv or scFv-Cmκ recombinant antibodies with patient sera for binding to cfc1-cyc conjugated to BSA (Figure 4). Competition (up to 81% reduction of the ELISA signal obtained by exchanging control sera antibodies with patient sera antibodies) was observed for all antibodies

Table 2. Reactivity of recombinant anti-CCP antibodies toward a panel of linear synthetic peptides[3]. X = Citrulline residue. Antibodies of patients (a, b, c) from which the recombinant antibody libraries were made (parent patient sera) showed similar peptide recognition patterns as the recombinant anti-CCP antibodies, whereas the control serum (Co) of a healthy individual showed no peptide reactivity. An anti-BSA recombinant antibody did not react with any of the peptides tested. Increasing signal strength denoted as + (0.2 < OD 450 nm < 0.4), ++ (0.4 < OD 450 nm < 0.7), +++ (0.7 < OD 450 nm < 1.0), ++++ (OD 450 nm > 1.0).

		Recombinant Antibody Clones																									
	Reactivity Groups →	1			2a										2b				3				αBSA	Patient Sera			
Peptide	Peptide Sequence	Ra3	A2-2	F12-2	B3-6	B5-4	B8-6	C1-2	G6-2	G7-6	H11-2	C3-4	D7-2	H2-2	B2-2	D3-4	A3-6	H9-4	D6-4	A8-6	G1-2	E1-6	BSA	Co	c	b	a
cfc1	SHQESTXGRSRGRSGRSGS	++++	++++	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	+++	+++	+++	++	-	-	++++	++++	+++
cfc2	SHQESTRGXSRGRSGRSGS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
cfc3	SHQESTRGRSXGRSGRSGS	+++	+++	+++	++++	++++	+++	+++	+++	+++	+++	++	++	+	++	++	++	+	-	-	-	-	-	-	++++	+++	+
cfc4	SHQESTRGRSRGXSGRSGS	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cfc5	SHQESTRGRSRGSGXSGS	+++	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cfc6	SHQESTXGRSRGRSGXSGS	+++	++++	+++	++++	++++	++++	+++	+++	+++	++++	+++	+++	+++	++++	+++	++	++	+	-	-	-	-	-	+++	+	-
cfc7	SHQESTXGXSRGRSGRSGS	++++	++++	++++	+++	+++	+++	++	+++	+++	+++	+++	++	++	++++	+++	++	+++	++	+	-	-	-	-	+++	+	-
cfc8	SHQESTXGRSXGRSGRSGS	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	+++	+++	++	++	+	+	+	-	-	++++	++	-
cfc9	SHQESTXGRSRGXSGRSGS	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++	-	+	-	-	-	+++	+	-
cfc1-319	SHQESTXGRSRGRS	++	++++	+++	++++	+++	++++	++++	++++	++++	++++	++++	+++	+++	++++	++	++	++	-	-	-	-	-	-	++	-	-
cfc1-318	SHQESTXGRSRGR	++	++	+++	+++	+++	++++	++	+++	++++	++++	++	++	++	++++	+	+	-	-	-	-	-	-	-	++	-	-
cfc1-317	SHQESTXGRSRG	++	++	+	++++	++++	++++	++	+++	+++	++++	++++	++	++	++++	++	+	-	-	-	-	-	-	-	+	-	-
cfc1-316	SHQESTXGRSR	++	++	+	+++	++++	++++	++	+++	+++	++++	+++	++	++	++++	+	+	+	-	-	-	-	-	-	+	-	-
cfc1-315	SHQESTXGRS	+	+	+	++	++	++++	-	+	+	++	+	++	-	+	-	-	-	-	-	-	-	-	-	-	-	-
cfc1-314	SHQESTXGR	-	-	-	+++	++	++++	++	++	+++	+++	+	++	-	++++	-	-	-	-	-	-	-	-	-	-	-	-
cfc309-316	ESTXGRS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cf0	SHQESTRGRSRGRSGRSGS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CfA	SHQESTAGRSRGRSGRSGS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CfE	SHQESTEGRSRGRSGRSGS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CfQ	SHQESTQGRSRGRSGRSGS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cf18-31	TGPSTRGRQGSXHE	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cf18-34-2	TGPSTRGXQGSRHEQAQ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cf18-34-3	TGPSTXGRQGSRHEQAQ	+	+	+	++	++	++	+	+	++	++	++	++	+	-	-	-	-	-	-	-	-	-	-	+	-	-

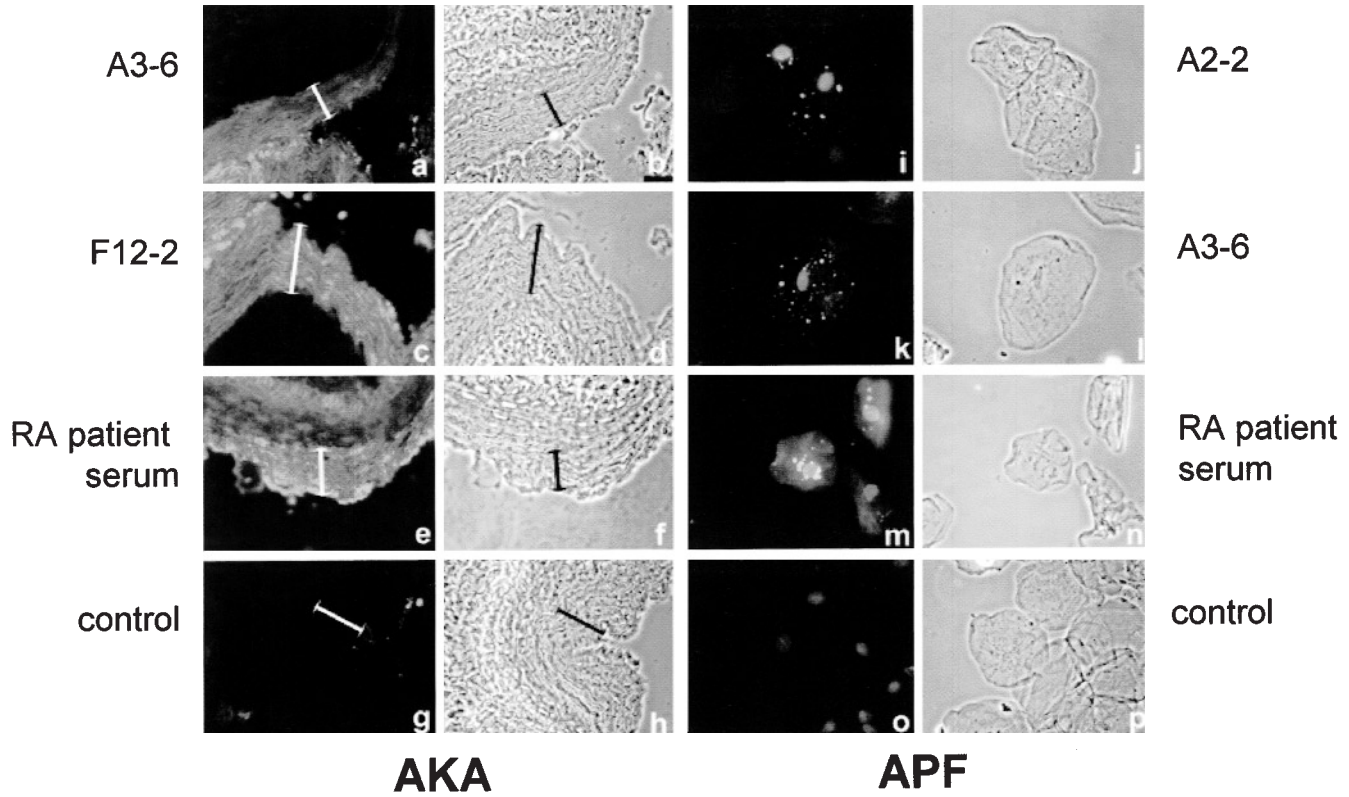

Figure 2. Antiperinuclear factor (APF) and antikeratin antibody (AKA) immunofluorescence tests using recombinant anti-CCP antibodies. APF: Human buccal mucosa cells are stained with soluble scFv or scFv-Cmκ fragments of individual recombinant antibodies. Perinuclear granules were stained with most recombinant antibodies, although intensity of staining varied strongly between the different recombinant antibodies. Only the staining patterns with clones A2-2 (panels i and j) and A3-6 (panels k and l) are shown. APF staining patterns of patient serum c (1:5 dilution) (panels m and n) were identical to the staining patterns obtained with recombinant anti-CCP antibodies. No staining was observed with control sera or with secondary antibodies only (panels o and p).
AKA: Rat esophagus sections were stained with soluble scFv or scFv-Cmκ fragments. Most recombinant antibodies showed staining of the stratum corneum. Staining patterns were similar to those obtained with the parent patient sera. Note that most recombinant antibodies show much lower background staining compared to the patient serum. Only staining patterns with clones A3-6 (panels a and b) and F12-2 (panels c and d) and with patient serum c (dilution 1:5) (panels e and f) are shown. No staining was observed with normal serum or with secondary antibodies only (panels g and h).

tested. For clones B3-6 and E1-6, competition was observed only when patient serum c or a mix (m) of 8 RA patient sera was used. For clone G1-2 competition was observed only with patient serum b or a mix (m) of 8 RA patient sera.

V gene usage. For the 21 clones analyzed, the nucleotide sequences of VH and VL genes were determined and compared with the germline sequences in the V BASE sequence directory (Tomlinson, *et al*, MRC Centre for Protein Engineering, Cambridge, UK[28-30]). Sequence analysis of the citrulline-containing peptide binders showed that they were derived from 3 VH families (representing 5 germline genes), one VLλ family (representing 4 germline genes), and one VLκ family (representing one germline gene). The results of the Clustal W alignments (amino acid level, using the software at http://www2.ebi.ac.uk/clustalw/ [cited March 12, 2003]; EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, Cambridge, UK) are shown in Tables 4 and 5.

VH gene analysis. The very high representation of VH4 germline gene 4-b (DP 67) was remarkable. Seventeen of 21 clones analyzed were derived from this germline gene and all contained highly homologous CDR3 regions. Based on the nucleotide and aa mutations these clones could be subdivided into 6 VH lineages. All 17 clones were extensively mutated throughout the whole VH region. Many mutations observed in the FR and CDR1 regions were conserved or semiconserved substitutions. For example, FR1 position 23 is mutated in all but one clone, and at position 31 in the CDR1 a semiconserved substitution is present in all but 3 clones. A hotspot of mutational activity (between aa 54 and 61) was present in CDR2 and to a lesser extent throughout FR3. The mutation of a tyrosine into a phenylalanine (position 58) in all but one clone is remarkable. In FR3 at position 68 a semiconserved substitution was present in all clones to either an N or an S, and both positions 78 and 82 contain conserved substitutions in many clones. Additional mutational hotspots were found at positions 82a, 85, and 89. Next to the germline gene 4-b derived recombinant antibody clones, one recombinant antibody derived from each germline gene 3-09, 3-21, 3-23, and 7-04.1 was obtained.

Table 3. Overview of recombinant antibody reactivities on peptide ELISA, Western blot, and immunofluorescence analyses (IFA). Strength of the signals is indicated in increasing order by – (negative), –/+, +, ++, +++, ++++ (strongly positive). Question mark indicates inconclusive result, ND: not determined.

Clone	Reactivity Group	Peptide ELISA	Western Blot				IFA	
		OD cfc1	Filaggrin	Citrullinated Fibrinogen			APF	AKA
				α	ß	200		
Ra3	1	++++	+++	+++	+	–/+	+++	+++
A2-2	1	++++	+++	+++	–/+	–/+	+++	+++
F12-2	1	++	+++	++	–/+	+++	+++	+++
B3-6	2a	+++	+	+++	–	+	+	+
B5-4	2a	++++	+++	ND	ND	ND	ND	ND
B8-6	2a	++++	+++	++++	?	–/+	++	+
C1-2	2a	++++	+	++++	–	+++	+++	+++
G6-2	2a	++++	+++	ND	ND	ND	ND	ND
G7-6	2a	++++	+	++++	–	+++	+++	+++
H11-2	2a	++++	+++	ND	ND	ND	ND	ND
C3-4	2a	++++	+++	ND	ND	ND	ND	ND
D7-2	2a	++++	–	++	–	–	–/+	+
H2-2	2a	+++	+	ND	ND	ND	ND	ND
B2-2	2b	++++	++	–/+	–	–	–/+	–/+
D3-4	2b	++++	+++	+	?	+	++	+++
A3-6	2b	++++	+	–/+	–	+++	++	+++
H9-4	2b	+++	+	ND	ND	ND	ND	ND
E1-6	3	++	–/+	–	–	+	–/+	+
D6-4	3	+++	+++	+	?	–	+	–/+
A8-6	3	+++	++	++	?	++	–/+	+
G1-2	3	++	–	+	–	–	+++	+

All contained many aa mutations (13 to 29), mainly concentrated in the CDR2 and FR3 regions. In general, recombinant antibodies derived from different germline genes showed different CDR3 regions. All but one VH gene were rearranged with relatively rare JH regions. Eighteen VH genes were rearranged with JH3. JH3 rearrangement normally occurs in about 6% in combination with VH3 and 10% with VH4 genes[33]. One JH2 rearrangement with a VH3 gene (normal occurrence 2%[33]) and one JH5 rearrangement (10% of all VH rearrangements[33]) with a VH7 gene were present. Finally, one VH3 gene was rearranged with the most frequently used JH4 region.

VL gene analysis. It has been reported by several groups that a large number of autoantibodies employ Vλ chains[34-36]. In accord with this observation is the preferential usage of Vλ1 derived light chain genes (20 out of 21 clones) we observed in the anti-CCP antibody fragments. More surprising was the observation that although these light chains were derived from different germline genes, their CDR3 regions are highly homologous. The number of aa mutations observed varied between 4 and 26, but they were on average less than the number of aa mutations observed in the VH genes. Mutations are present throughout the whole V region, although higher mutation levels were observed in the CDR1 and the CDR2 regions. The 2 clones derived from the germline gene 1a (Ra3 and E1-6) are derived from 2 different B cell lineages. The same is true for clones derived from germline 1b, although clones G1-2 and A2-2 are more closely related to each other than to clone F12-2. Based on the homology of their CDR3 regions and the position of aa mutations throughout the VL region, clones derived from germline 1c were divided into 6 groups. No remarkable VL rearrangements were observed. Both the observed JL2 and JL3 are used frequently in the normal repertoire, and the Vλ1 family is the Vλ family most frequently used in the normal repertoire (for an overview see Tables 4B and 5). Clones A8-6 and C1-2 derived from germline gene 1g are near germline sequence, whereas clone H11-2 derived from the same germline gene is highly mutated (19 aa mutations). Only one Vκ antibody was obtained that used germline gene 012 of the Vκ1 family rearranged with JK1.

We compared the peptide reactivity groups with the genetic relationship of the antibody clones. A strong bias was observed between clones belonging to specific reactivity groups identified by ELISA and their genetic origins. Reactivity group 1 was represented by antibody clones derived from VH germline genes VH3 3.23 (Ra3) VH3 3-21 (A2-2), and VH7 7-04.1 (F12-2). Reactivity groups 2 and 3 are all represented by closely related germline gene 4-b derivatives and one germline gene VH3-09 derived clone (D7-2). It is notable that VH7 7-04.1 and the highly represented VH4 4-b are derived from VH alleles that are not present in all individuals (V BASE sequence directory; Tomlinson, *et al*, MRC Centre for Protein Engineering). An overview of reactivity groups, germline gene usage, CDR 3 sequences, and mutations present in all clones is given in

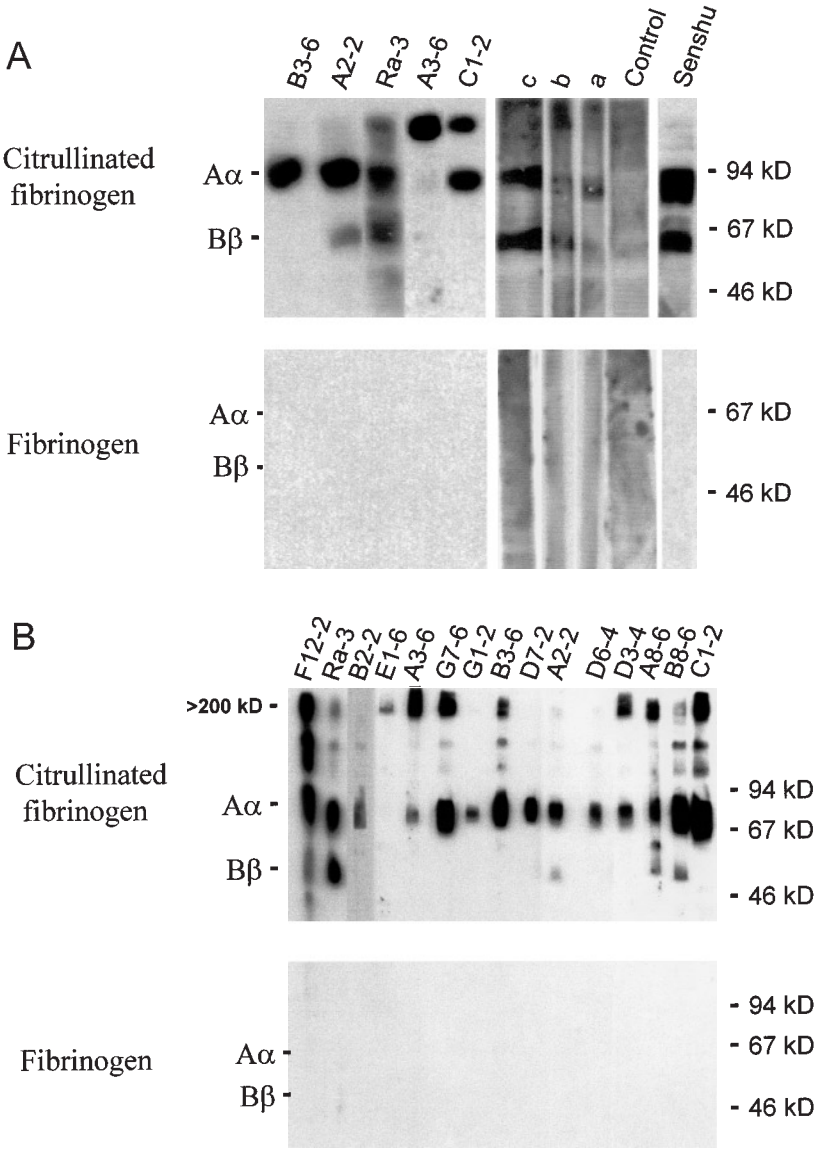

Figure 3. Western blots containing fibrinogen and citrullinated fibrinogen incubated (A) with some recombinant anti-CCP antibodies, patient sera a, b and c, and the citrulline-specific Senshu antibody; and (B) with a panel of recombinant anti-CCP antibodies. Most recombinant antibodies stained the citrullinated Aα subunit of fibrinogen, whereas some clones stained the Bß subunit as well. Many clones also cross-reacted with a high molecular weight band (> 200 kDa). Positions of Aα and Bß chains were determined by Ponceau S staining of the blots before incubation with antibodies.

Table 5. To rule out that the strong bias toward VH4 4-b usage and the very high frequency of Vλ1 derived antibodies with highly homologous CDR3 regions was caused by a bias already present in our RA patient libraries, 8 noncitrulline-specific clones selected from the same RA libraries were analyzed as well (overview, Table 5).

DISCUSSION

Recently it was reported that detection and quantitation of anti-CCP antibodies in patients with RA provide an excellent serological test for the early diagnosis of RA[4]. Currently, the only targets for these antibodies in RA patient synovia have been the citrullinated forms of α and/or ß fibrin(ogen)[8].

We isolated and characterized 21 different recombinant human anti-CCP autoantibodies from phage display antibody libraries derived from patients with RA.

The recombinant antibodies. The recombinant antibodies analyzed displayed 3 different reactivity patterns based on their recognition of subsets of linear peptides. All antibody

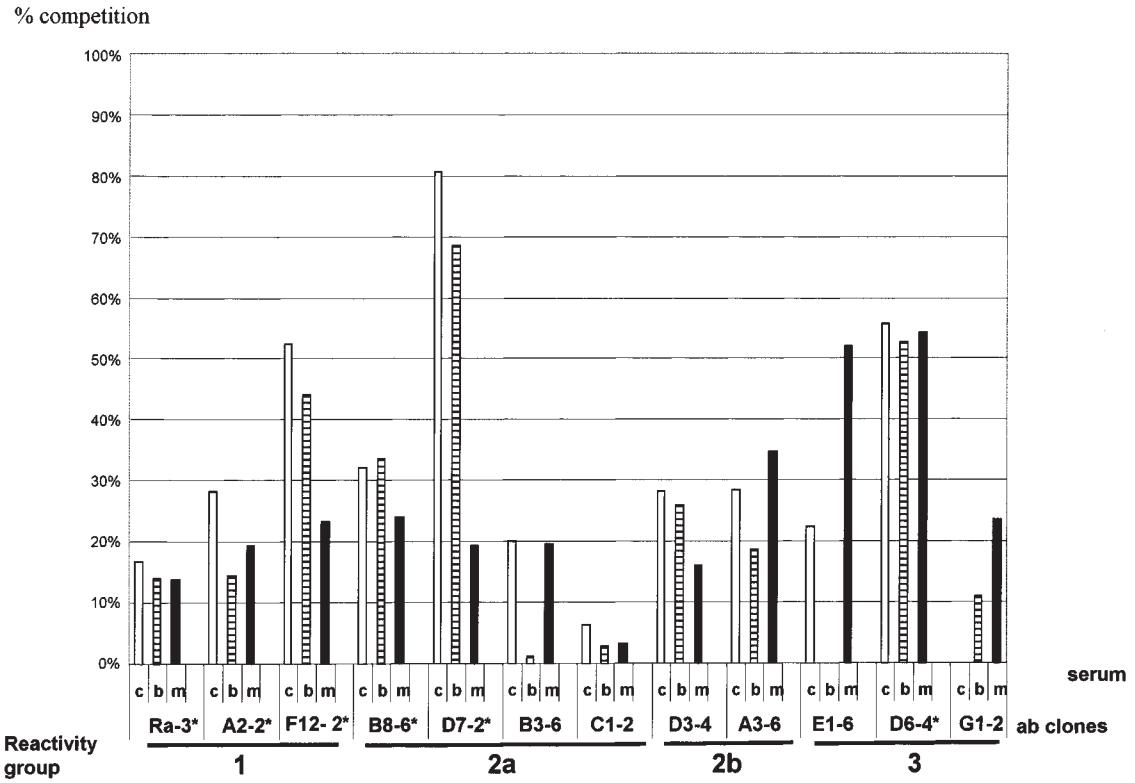

Figure 4. Competition ELISA of scFv with patient serum. Competition was analyzed for binding to peptide cfc1-cyc conjugated to BSA, with a panel of recombinant anti-CCP antibodies, RA patient sera, and noncompeting control sera. The various recombinant antibodies were premixed with a serial dilution of the competing patient serum: serum b or c or a mix (m) of 8 randomly selected RA patient sera. Equal amounts of noncompeting control serum were used as a negative control. Recombinant antibodies were used in a dilution resulting in an OD 450 of 2/3 of the maximum OD in the linear range of the binding curve of the recombinant antibodies, as determined by endpoint titration. Percentage competition was plotted. The OD 450 signal of the blank (no recombinant antibody added) was defined as 100% inhibition and the signal with noncompeting control serum added to the incubation was defined as 0% inhibition. Competing antibodies for clones F12-2, D7-2, and D6-4 were present at high concentration in the individual parent patient sera tested. Antibody clones E1-6 and D6-4 are also present at high concentrations in nonrelated RA sera. Note the large variation in competition observed for clones B3-6 and E1-6 (both present in serum c and in the RA mix, but not in serum b) and for G1-2 (present in serum b and RA mix, but not in serum c). *scFv-Cmκ fragments; other recombinant antibodies were used in the scFv format.

clones exclusively recognized peptides containing a citrulline residue(s). Clone Ra3 appeared to be the least selective for the peptide context in which the citrulline is present. Most peptides harboring a citrulline residue were recognized by this clone (although the reactivity with the different citrullinated peptides varied strongly). An increased selectivity for the peptide context of citrulline was observed for the other clones. All selected antibodies were also analyzed by APF and AKA immunofluorescence based tests, and all were positive in these tests, confirming that

Table 4. Alignment of VH (Table 4A) and VL (Table 4B) chains to their most homologous germline genes. Consensus lines derived from clustal W alignments are indicated by CWx*. Table 4A: CWa = alignment of germline 4-b derived genes. CWa* = alignment of germline gene 4-b derived genes without their germline gene. CWb = alignment of germline gene 3-09 derived genes. CWc = aligment of germline gene 3-21 derived genes. CWd = alignment of germline gene 3-23 derived genes. CWe = alignment of germline 7-04.1 derived genes. CWH3T* = alignment of all VH3 derived genes without their germline genes. Table 4B: CWa = alignment of germline 1a derived genes. CWb = alignment of germline 1b derived genes. CWc = alignment of germline gene 1c derived genes. CWd = alignment of germline gene 1g derived genes. CWT* = alignment of all Vλ1 derived genes without their respective germline genes. CWe = alignment of germline 012 derived genes. The clustal W consensus lines display the following symbols denoting the degree of conservation observed in each column: "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, according to (AVFPMILW = Small (small + hydrophobic (incl. aromatic –Y); DE = Acidic; RHK = Basic; STYHCNGQ = Hydroxyl + Amine + Basic – Q), "." means that semiconserved substitutions are observed. C/S/N = number of conserved/semiconserved/nonconserved mutations in FR1/2/3 and CDR 1/2 regions. For the sequence alignments, dots indicate identical residues, dashes indicate gaps, small bold type indicates regions introduced by oligonucleotides used for amplifications of the VH and VL regions. Framework and CDR regions are indicated by FR1, FR2, FR3, FR4, CDR1, CDR2, and CDR3. Germline genes are underlined. Reactivity groups are indicated by the numbers 1–3. For an overview of the genetic analyses see Table 5.

Table 4A

Clone	React. group	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	C/S/N
		1 2 3		4	5 6	7 8 9			
		123456789012345678901234567890	1ab2345	67890123456789	012abc3456789012345	67890123456789012abc345678901234			
VH4 4-b		qvqlqesGPGLVKPSETLSLTCAVSGYSIS	SG-YYWG	WIRQPPGKGLEWIG	SIY---HSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR			
H11-2	2a	e...v.................V..AS...	D.-....	.......R.....A	..F---RN.Y.F..S....	..N......Q.......H......A.T.....	GLSIYGPDD------AFAL	WGQgtmvtvsts	6/8/5
C3-4	2a	e...v.................V..AS...	D.-....	.......R.....A	..F---RN.Y.F..S....	..N......Q.......H......A.T.....	.........------....	WGQgtmvtvsts	6/8/5
B8-6	2a	e...v.................V..AS...	D.-....	.......R.....A	..F---RN.Y.F..S....	..N......Q.......H......A.T.....	.........------....	WGQgtlvtvsts	6/8/5
H2-2	2a	............V......I.D.....MT	G.-....	.............A	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	GLRISGISD------AFEI	WGQgttvtvsts	8/5/11
A3-6	2b	............V......I.D.....MT	G.-....	.............A	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	.........------....	WGQgtmvtvsts	9/5/11
B3-6	2a	.....q......V......I.D.....MT	G.-....	.............A	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	.........------....	WGQgtmvtvsts	9/5/12
B5-4	2a	....vq......V......I.D.....MT	G.-....	.............A	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	.........------....	WGQgtmvtvsts	10/5/11
H9-4	2b	.....q.....RV......I.D.....MT	G.-....	.............A	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	.........------....	WGQgtlvtvsts	9/4/11
G6-2	2a	.....q......V......I.D.....MT	G.-....	....	.TS---.RMYSFH.E....	..N..I.P....L...MY....E...T.....	....F....------....	WGQgtmvtvsts	
D3-4	2b	.....q................V..NS...	N.-....	.L..A.........	...---PNINDF..T....	..N....P...HL..TMT....S...M.F...	GLSHFGTND------AFAF	WGQgtmvtvsts	10/10/4
C1-2	2a	......................V..NS...	N.-....	.L..A.........	...---PNRNDL..T....	..N........HL..TMTY...S...M.F...	.........------....	WGQgtmvtvsts	8/9/6
G7-6	2a	.....q................V..NS...	..-....	.M............	...---PNRNDF..T....	..N........HL...MT....S...I.F...	....Y....------..EV	WGQgtmvtvsts	10/6/4
B2-2	2b	e...v.....................F.VR	N.-....	.L............	..W---.DRDSF..T....	..N.A..S....L..QMT....S...I.F...	....Y....------..EV	WGQgtmvtvsts	16/5/3
E1-6	2b	......................N.......	..-FF..	....	..H---.D.R.F..S..ET	..SL.......H...RM.G.S.T.......W.	GRSRSGPND------AFEI	WGRgtmvtvsts	12/3/4
G1-2	3	.....q................I...D...	..-....	....S.........	...---.DRR.F..S..E.	..SL.......H...RM.G.S.T...I...S.	.........------....	WGQgtmvtvsts	10/4/6
D6-4	3	e...v....V............N...D...	D.-....	.......R......	.V.---YNRN.F..S..E.	..SL.L.....HL..TM.D.........F.S.	...KF....------....	WGQgtmvtvsts	15/4/6
A8-6	3	.............S........H.......	D.-....	....S.........	.RH---.G.NA.F....H.	..SLLI......L...MH........I.....	GLHIDGWND------AFEI	WGRgtmvtvsts	9/4/6
CWa		:*** :****:*: ****** * ** *:	.* ::**	*:**.**:*****.	* :* * ::	**.: :*.*:*::** : *:* *:* *:* *			
CWa*		:*** :****:*: ****** * ** *:	.* ::**	*:**.**:*****.	* ::* * ::	**.: :*.*:*::** : *:* *:* *:* *	* * .* ** .	**:** ******	
VH3 3-09		evqlvesGGGLVQPGRSLRLSCAASGFTFD	D--YAMH	WVRQAPGKGLEWVS	GISW--NSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAK			
D7-2	2a	....	.--....	....S........A	..N.--DG.D.....R.R.	..........K....R.D...VD.......V.	PDYYDVSGYGWY----FDI	WGRgtlvtvsts	8/5/1
CWb		******************************	* ****	****:********:	**.* :.*.***** *:*	**********:****:*:***.:*******.*			
VH3 3-21		evqlvesGGGLVKPGGSLRLSCAASGFTFS	S--YSMN	WVRQAPGKGLEWVS	SISS--SSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR			
A2-2	1	q...qq......R..............NL.	T--NF..	....SR......I.	...W--TGDD..E...L..	...V.......TV...LS..TPD.........	VR-QYRDGRGYVVNDALDI	WGQgtmvtvsts	14/5/8
CWc		:*** :******:**************.:*	: **	****: ******:*	*** :.. ** ***:**	***:*******::***:.** .:*********			
VH3 3-23		evqllesGGGLVQPGGSLRLSCAASGFTFS	S--YAMS	WVRQAPGKGLEWVS	AISG--SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK			
Ra3	1	q.n.r......A.............E...G	H--....	....	G...--D.TGK..TA....	..N..K.H....MT...D...V....T...T.	DS-YTRWG-KWYP---IDH	WGQgtlvtvsts	8/9/6
CWd		:*:* ******.************* ***.	****	**************	.*** .* ..**: ****	**.**:*:****: ***:***.****.***:*			
CWVH3T*		:*:* :*****.:** ********* *.:.	*	****: ******::	.*. * . : ::*	**.:*:*::*::: *::.** :*** ***.:	* : :*	**:**:******	
VH7 7-04.1		qvqlvqsGSELKKPGASVKVSCKASGYTFT	S--YAMN	WVRQAPGQGLEWMG	WINT--NTGNPTYAQGFTG	RFVFSLDTSVSTAYLQICSLKAEDTAVYYCAR			
F12-2	1	....q...............I.......SL.	D--.TI.	....	....--K...S....D.I.	....A............S........L.....	GR-YSLTRFD--------P	WGQgtlvtvsts	8/4/2
CWe		**** **************:*******::*	. *::*	**************	**** :***.****.* *	****:************.********:*****			

Table 4B

Clone	React. group	FR1	CDR1	FR2	CDR2	FR3	CDR3		C/S/N
		2	3	4	5	6 7 8	9		
		12345678912345678901234567890123	45678901abc234	567890123456789	01abcde23456	7890123456789ab90123456789012345678	9012345abcde		
Vλ1 1a		qsvltqpPSVSEAPRQRVTISC	SGSSSNIGNN-AVN	WYQQLPGKAPKLLIY	YD-----DLLPS	GVSDRFSGSKSG--TSASLAISGLQSEDEADYYC	AAWDDSLNG	----------	
Ra3	1	sy.....	.....-...	.....R.....	..-----.....	.....--.....	.....-VV	FGGgtkltvlg	1/1/1
E1-6	2b	sy.....G...N...	...R....Y.-...	....VA.....M.	..------SV...	.....--.....	.V...R.D.-WV	FGGgtkltvlg	4/4/4
CWa		. ********** ***.***	*** **** * ***	****:.*:*****:*	** .:***	********** **********	*.*** *:* :*	**********	
Vλ1 1b		qsvltqpPSVSAAPGQKVTISC	SGSSSNIGNN-YVS	WYQQLPGTAPKLLIY	DN-----NKRPS	GIPDRFSGSKSG--TSATLGITGLQTGDEADYYC	GTWDSSLSA	----------	
F12-2	1	.....S.....	...N....I.-...	.....	..-----.....	.....--.....	....N....-WV	FGGgtkltvlg	0/2/1
G1-2	3	.....	.....-.A.	.....	G.------.N...	.V.....--...S.A.S...SE.....	AA..D..NG-WV	FGGgtkltvlg	5/3/1
A2-2	1	.....M.GT.....	.....-..Y	....V.....	.D-----.....	.V.....--.....R...S.....	AA..D..NG-VV	FGGgtkltvlg	6/1/3
CWb		*******.*:*.:*********	***.**** * *.	****:**********	.: *:***	*:********** ***:*.* ***: *******	.:**.**.. .*	**********	
Vλ1 1c		qsvltqpPSASGTPGQRVTISC	SGSSSNIGSN-TVN	WYQQLPGTAPKLLIY	SN-----NQRPS	GVPDRFSGSKSG--TSASLAISGLQSEDEADYYC	AAWDDSLNG	----------	
C3-4	2a	.....	.....-...	.....	..-----D....	.....--.....	.....-WV	FGGgtkltvlg	0/1/0
B5-4	2a	.....S.....A...	...T.....-...	.....M.	..------I....	.....--.....	.....-WV	FGGgtkltvlg	3/2/0
H2-2	2a	.....I...	.....-Y..	.....H	T.-----.....	.....--.....	.....-WV	FGGgtkltvlg	1/1/2
H9-4	2b	sy.....	T.....-...	.....	.-----.....	.....--.....	.....-WV	FGGgtkltvlg	1/1/1
A3-6	2b	sy.....	.....-...	.....	RS-----D....	.....--..S.....R.....	.....D.-WV	FGGgtqltvlg	3/2/2
D6-4	3	.....	.....-P.T	.....A....V..	.D-----.E...	..A.....--.....V.....	.....SWV	FGGgtkltvlg	3/2/3
B3-6	2a	hvi....S.....	.....RP.-S..	...H.....I.H	DD-----.....	.....--.....D.....	.....-WV	FGGgtkltvlg	7/2/4
G6-2	2a	hv.....S.....	.....R.-S..	...H.....I.H	N.-----.....	.....--.....	.....-WV	FGGgtkltvlg	4/2/3
G7-6	2a	sy.....V.....	..HR.....-N..	.....	..-----.R...	.....--.....	SS..N....-WV	FGGgtkltvlq	1/3/3
B8-6	2a	.....	...D.D...H-K..	...H.....L.	.D-----D....	.....A..--.....G....	SV...T.R.-WV	FGGgtkvtvlg	5/3/1
B2-2	2b	.....V.....	..GG....T.-R.Q	...KVA.....M.	ED-----DE...	.....M.D--.....GE...	S.....FR.-WA	FGGgtkltvlg	9/6/3
D3-4	2b	hvi....S...E....S.....	.....R.-P.I	..H.I..M..R..VH	TT-----D....	.....R..--.....T.....GE.F.	S....NV..-WV	FGGgtkltvlg	12/3/8
CWc		:****.*.* **** * ***	:* *:* : *	**:::.* **:*:::	.***	*** ***** *. **:**.****:*:**.:*:*	: **:.. * *.	*****::****	
Vλ1 1g		qsvltqpPSASGTPGQRVTISC	SGSSSNIGSN-YVY	WYQQLPGTAPKLLIY	RN-----NQRPS	GVPDRFSGSKSG--TSASLAISGLRSEDEADYYC	AAWDDSLSGP	----------	
A8-6	3	.....	.....-F..	.....N	S.-----.....	.....--.....	.....-WM	FGGgtkvsvlg	1/0/2
C1-2	2a	.....V.....	..HR.....-N.N	.....	..-----.....	.....--.....	.....-WV	FGGgtqltvls	0/1/4
H11-2	2a	.....V.....	F..D....Y.-N.I	....V.....H	TS-----D....	.....--.....	.S.H.D.G.FWV	FGGgtkvtvlg	2/3/6
CWd		********.**********	* **** * *	****:*********	. :****	********** **********	*:*.*.*.* *	*****:::**.	
CWT*		:****.*.* :*** * ***	* *:* : .	**:::.* **:*::	. **	**. ***** *. **:**.****:*:**.:*:*	: *.: . * :	*****:::**.	

		FR1	CDR1	FR2	CDR2	FR3	CDR3		
		1 2	3	4	5	6 7 8	9		
		12345678901234567890123	45678901abcdef234	567890123456789	0123456	7890123456789012345678901234567890	9012345		
VκI O12		diqmtqsPSSLSASVGDRVTITC	RASQSISS------YLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	----------	
D7-2	2a	.....	.....TT------...	....R.....N....	.....	.....R.....	.....---RT	FGQgtkleikr	4/0/1
CWe		**********	******:: ***	****:*****:****	*******	****** **********	*******		

Table 5

Anti-CCP clones	react. group	VH germline gene	CDR3	mutations[A] aa	mutations[A] nu	VL	germline gene	CDR3	mutations[A] aa	mutations[A] nu	VH / VL DJ / J
Ra3[b]	1	VH3 3-23 (DP-47)	DSYTRWGKWYPIDH	20 (3)	43 (8)	Vλ1	1a (DPL1)	AAWDDSLNG-VV	4 (2)	3 (1)	D4-b JH4b / JL2/JL3a
A2-2[c]	1	VH3 3-21 (DP-77)	VRQYRDGRGYVVNDALDI	24 (3)	38 (6)	Vλ1	1b (DPL5)	AAWDDSLNG-VV	18	33 (1)	D1-20 JH3b / JL2/JL3a
F12-2[c]	1	VH7 7-04.1 (VI-4.1b)	GRYSLTRFDP	13 (1)	22 (5)	Vλ1	1b (DPL5)	GTWDNSLSA-WV	7	5 (3)	JH5b / JL3b
D7-2[c]	2a	VH3 3-09 (DP-31)	PDYYDVSGYGWYFDI	14	26 (1)	VκI	O12 (DPK9)	QQSYSTP---RT	8	11	D3-22 JH2 / JK1
H11-2[c]	2a	VH4 4-b (DP-67)	GLSIYGPDDAFAL	17 (2)	43 (4)	Vλ1	1g (V1-17)	ASWHDDLGGFWV	19	39 (3)	JH3a/JH3b / JL3b
B8-6[c]	2a	VH4 4-b (DP-67)	GLSIYGPDDAFAL	17 (2)	43 (4)	Vλ1	1c (DPL2)	SVWDDTLRG-WV	17	28 (3)	JH3a/JH3b / JL3b
C3-4[c]	2a	VH4 4-b (DP-67)	GLSIYGPDDAFAL	17 (2)	42 (4)	Vλ1	1c (DPL2)	AAWDDSLNG-WV	4	4 (4)	JH3a/JH3b / JL3b
B5-4[c]	2a	VH4 4-b (DP-67)	GLRISGISDAFEI	24 (2)	50 (4)	Vλ1	1c (DPL2)	AAWDDSLNG-WV	8	9 (1)	JH3b / JL3b
B3-6[c]	2a	VH4 4-b (DP-67)	GLRISGISDAFEI	24 (1)	47 (3)	Vλ1	1c (DPL2)	AAWDDSLNG-WV	13 (3)	16 (8)	
H9-4[c]	2b	VH4 4-b (DP-67)	GLRISGISDAFEI	25 (1)	49 (3)	Vλ1	1c (DPL2)	AAWDDSLNG-WV	4 (2)	6 (4)	JH3b / JL3b
H2-2[c]	2a	VH4 4-b (DP-67)	GLRISGISDAFEI	24	47	Vλ1	1c (DPL2)	AAWDDSLNG-WV	7	9 (3)	JH3b / JL3b
A3-6[c]	2b	VH4 4-b (DP-67)	GLRISGISDAFEI	24	49	Vλ1	1c (DPL2)	AAWDDSLDG-WV	9 (2)	13 (4)	D1-7 JH3b / JL3b
G6-2[c]	2a	VH4 4-b (DP-67)	GLRIFGISDAFEI	23 (1)	49 (3)	Vλ1	1c (DPL2)	AAWDDSLNG-WV	10 (2)	9 (6)	JH3b / JL3b
D3-4[c]	2b	VH4 4-b (DP-67)	GLSHFGTNDAFAF	23 (1)	48 (3)	Vλ1	1c (DPL2)	SAWDDNVNG-WV	26 (3)	35 (8)	JH3a/JH3b / JL3b
C1-2[c]	2a	VH4 4-b (DP-67)	GLSHFGTNDAFAF	23	47	Vλ1	1g (V1-17)	AAWDDSLSG-WV	8	11 (3)	JH3a/JH3b / JL3b
G7-6[c]	2a	VH4 4-b (DP-67)	GLSHYGTNDAFEV	19 (1)	39 (3)	Vλ1	1c (DPL2)	SSWDNSLNG-WV	11 (2)	11(4)	JH3a / JL3b
B2-2[c]	2b	VH4 4-b (DP-67)	GLSHYGTNDAFEV	22 (2)	39 (4)	Vλ1	1c (DPL2)	SAWDDSFRG-WA	24	42(3)	D2-8 JH3a / JL3b
A8-6[c]	3	VH4 4-b (DP-67)	GLHIDGWNDAFEI	19	40	Vλ1	1g (V1-17)	AAWDDSLSG-WM	6	8 (3)	JH3b / JL3b
E1-6[c]	3	VH4 4-b (VHSP)	GRSRSGPNDAFEI	19	40	Vλ1	1a (DPL1)	AVWDDRLDG-WV	15 (2)	20 (1)	JH3b / JL3b
G1-2[c]	3	VH4 4-b (VHSP)	GRSRSGPNDAFEI	19 (1)	39 (3)	Vλ1	1b (DPL5)	AAWDDSLNG-WV	17	29 (1)	JH3b / JL3b
D6-4[c]	3	VH4 4-b (VHSP)	GRSKFGPNDAFEI	23 (2)	43 (4)	Vλ1	1c (DPL2)	AAWDDSLNGSWV	12	20 (3)	JH3b / JL3b

Anti-nonCCP clones	VH germline gene	CDR3	mutations[A] aa	VL	germline gene	CDR3	mutations[A] aa
Ra1[a]	VH3 3-33 (DP-50)	DQEVRYDILTGWAPSGSYYYYYMDV	5 (1)	Vλ3	31 (DPL-16)	NSRDSRGNHLV	6 (1)
Ra2[a]	VH4 4-04 (DP-70)	DRAPDYDIVTGYPNTGRRFDP	12 (1)	VκII	A19(DPK-15)	MQGQLQTPLT	5 (1)
Ra4[b]	VH7 7-04.1 (Vi-41.b)	GKVAGDHWYFDL	2 (2)	Vλ3	31 (DPL-16)	NSRDSSGKHVV	11 (1)
Ra5[b]	VH3 3-49	GRGLLDY	16 (2)	VκI	L12(L12A)	QQYNRYPLT	12
Ra6[b]	VH1 1-46 (DP-7)	GGSTAAKYFFAMDV	16	VκI	A30	QQHIAYPIT	15
Ra7[b]	VH1 1-46 (DP-7)	DRGLGYCSGGTCQRGFDI	8 (1)	Vλ1	1g (DPL-3)	AAWDDRMGWV	13
Ra8[b]	VH3 3-30 (DP-49)	DFRRGGYEDDGFDT	11 (2)	Vλ1	1b (DPL-5)	WDSSLSARV	8
Ra9[b]	VH3 3-30 (VHCOS-8)	DKRKKGRDFWTGYAYYYYGMDV	9 (2)	Vλ3	31 (DPL16)	NSRDTSGNHRV	9 (1)

Table 5. V gene family, germline derivation and CDR3 domains, (amino acid) mutations, and reactivity groups of anti-CCP scFv clones and non-CCP reactive scFv clones derived from RA libraries. Data in parentheses: additional oligo-induced substitutions.
[A]Mutations: For VH genes, the amino acid substitutions outside the CDR3 region are given, for VL genes all amino acid substitutions are listed.
[a]Selected with peptide P15 (noncitrulline-containing peptide).
[b]Selected with peptide cfc1-cyc.
[c]Selected with cfc1-cyc-biotin.
|: Genetic relationships are indicated by vertical bars.

they react with the citrullinated filaggrin protein that had been shown to be the antigen in these tests[3,10]. The reactivity with citrullinated filaggrin was also confirmed by Western blotting (data not shown). Recently, it was described that among the synovial targets of RA-specific autoantibodies were the citrullinated forms of the α and ß chains of fibrin(ogen)[8]. To determine whether the recombinant anti-CCP antibodies were also reactive with these proteins, we performed Western blotting with *in vitro* citrullinated fibrinogen and noncitrullinated fibrinogen. All recombinant antibodies tested were reactive with the Aα and/or the Bß subunit of citrullinated fibrinogen. As well we observed reactivity toward a high molecular weight band (> 200 kDa) reported to appear during citrullination of fibrinogen *in vitro* and reactive with antibodies against Aα-fibrinogen subunit[8]. In general, reactivity with the Aα subunit of fibrinogen was strongest. Finally, no reactivity with components of HeLa or HEp-2 cells was observed in immunofluorescence or Western blot assays. All our findings indicate that the recombinant antibody fragments selected in this study from RA patient derived phage display libraries are very specific for citrullinated targets and show no detectable cross-reactivity with noncitrullinated proteins.

Comparison of recombinant antibody and patient sera reactivity. Schellekens, *et al*[3] described that the autoantibody reactivity pattern of RA patients toward citrullinated peptides is specific and also very heterogeneous. Every RA serum appears to contain a large set of antibodies with slightly different specificities toward citrulline and its neighboring amino acids.

The variety in monoclonal antibody fragments selected in this study corroborates this idea. Indeed, the overall peptide reactivity pattern of the various recombinant human antibodies was very similar to the reactivity pattern observed in the respective patient sera. For both the recombinant antibodies and the patient sera, strong reactivity with the same peptides was observed. As well, in the APF and AKA tests we observed that all recombinant antibodies displayed similar staining patterns compared to RA patient sera (staining of the perinuclear granules in the APF test and staining of the stratum corneum in the AKA test). It has been shown[8] that, like the majority of recombinant antibody clones, most RA patient sera were reactive with citrullinated filaggrin and fibrinogen proteins on Western blot. To prove that the recombinant antibodies indeed bind to identical or overlapping epitopes as the patient antibodies, competition experiments with polyclonal human RA sera were performed. The results clearly showed that patient serum antibodies efficiently compete with recombinant antibodies for binding to the cfc1-cyc peptide. For some clones, competition was only observed when a specific patient serum or the combination of 8 RA patient sera was used. Thus, either the antibodies recognizing these epitopes are less common in RA sera or the recombinant antibodies recognizing these epitopes bind with higher affinity to these epitopes, making it more difficult for patient antibodies to compete for binding. Competition levels between 0 and 81% were observed. The wide range of competition is most likely caused by differences in the concentration of specific serum antibodies and/or possible differences in affinity (or avidity) of the recombinant antibodies. As expected, no competition was observed with sera derived from healthy individuals. All these data suggest that we have cloned a number of autoantibody fragments that are specifically present in the sera of patients with RA.

V gene usage. Various reports describe V gene usage in autoantibodies. Autoantibody VH gene frequencies comparable to those observed in the normal repertoire have been reported[37], as well as sequences that were very different[36]. Our group showed that preferential VH gene usage seems to occur when one considers only one particular antigen[19,23,38], and that VH genes with low frequency in the normal repertoire were used in some autoantibodies derived from libraries from patients with systemic lupus erythematosus[39]. All but one of the anti-CCP autoantibodies analyzed in this study were derived from the most common V gene families (VH3, VH4, Vλ1, and Vκ1). A few reports describe a prevalence of VH4 family rearrangements in RA plasma cells[40] or a selected humoral immune response encoded by VH4 family genes[41]. We also observed a strong bias for the VH4 family germline gene 4-b rearrangement with the (in the normal repertoire less frequently used) JH3 (a/b) regions (17 out of 21 clones with highly homologous CDR3 regions).

It has also been reported that many autoantibodies employ Vλ chains[34,36,42]. A strong bias toward usage of Vλ light chains was observed in the selected anticitrulline clones. The Vλ1 family (20 out of 21 clones) rearranged with J region JL3b (18 out of 20 clones) was used in the majority of the clones. Nearly all these clones displayed highly homologous CDR3 regions. The restricted usage of both VH and VL germline genes and their CDR3 homology

seems to suggest an important role for these V genes in citrullinated antigen recognition. That some VH alleles used (including the most predominant one) are not present in all individuals may indicate the existence of a genetic predisposition for the development of anticitrulline antibodies in individuals with these germline alleles. Sequence analyses of antibodies not recognizing citrulline-containing peptides (non-CCP antibodies) obtained in selections with the same RA patient libraries revealed that these antibodies used a different subset of VH and VL germline genes compared to the anti-CCP recombinant antibodies. Thus, we can rule out that the restricted germline gene usage in the anti-CCP antibodies was caused by a bias already present in the RA patient phage display libraries.

The majority of VH and VL genes contained a high level of mutations, which indicates an antigen driven response. Similar observations have been reported for other autoantibodies. V gene sequences derived from diseased synovium showed preservation in the framework regions and divergence in the CDR regions. Further, clonally related V gene sequences have been described in the diseased synovium[43]. All these observations suggest that these autoantibodies are generated in the patient as the result of the occurrence of (several) citrullinated antigens.

The availability of human recombinant monoclonal anti-CCP antibodies provides a unique tool to study and identify their citrullinated targets. Knowledge of these autoantigens will enable the design of novel tools for early diagnosis of RA and hopefully will help to unravel the etiology of this enigmatic disease.

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