Arthritis-related B Cell Epitopes in Collagen II Are Conformation-dependent and Sterically Privileged in Accessible Sites of Cartilage Collagen Fibrils*

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Stefan Schulte‡§, Christine Unger‡§§, John A. Mo¶, Olaf Wendler‡§§, Eva Bauer‡§§, Svenja Frischholz§, Klaus von der Marks§, Joachim R. Kalden¶, Rikard Holmdahl¶, and Harald Burkhardt‡§§

From the ¶Department of Internal Medicine III, Institute of Clinical Immunology and the §Institute of Experimental Medicine, University of Erlangen-Nürnberg, D-91054 Erlangen, Germany, and the §Section for Medical Inflammation Research, Department of Cell and Molecular Biology, Lund University, S-221 00 Lund, Sweden

In collagen-induced arthritis, a murine autoimmune model for rheumatoid arthritis, immunization with native but not heat-denatured cartilage-specific collagen type II (CII) induces a B cell response that largely contributes to arthritogenicity. Previously, we have shown that monoclonal antibodies established from arthritis-prone DBA/1 mice require the triple-helical conformation of their epitopes for antigen recognition. Here, we present a novel approach to characterize arthritis-related conformational epitopes by preparing a panel of 130 chimeric collagen X/CII molecules. The insertion of a series of CII cassettes into the triple-helical recombinant collagen X allowed for the first time the identification of five triple-helical immunodominant domains of 5–11 amino acid length, to which 75% of 36 monoclonal antibodies bound. A consensus motif, “RG hydrophobic,” was found in all immunodominant epitopes. The antibodies were encoded by a certain combination of V-genes in germline configuration, indicating a role of the consensus motif in V-gene selection. The immunodominant domains are spread over the entire monomeric CII molecule with no apparent order; however, a highly organized arrangement became apparent when the CII molecules were displayed in the quarter-staggered assembled assembly within a fibril. This discrete epitope organization most likely reflects structural constraints that restrict the exposure of CII epitopes on the surface of heterotypically assembled cartilage fibrils. Thus, our data suggest a preimmune B cell selection process that is biased by the accessibility of CII determinants in the intact cartilage tissue.

Rheumatoid arthritis is the most common chronic inflammatory joint disease in humans. The disease is genetically linked to the MHC-II region (1) and characterized by relapsing inflammation of synovial tissue and progressive destruction of cartilage and subchondral bone. The driving force of this disorder is still obscure. However, immune responses toward cartilage-specific antigens, particularly B cell responses against type II collagen (CII), indicate a pathogenic role of cartilage-specific autoimmunity (2–5).

CII, the predominant collagenous component of cartilage, is one of the candidate autoantigens potentially fueling tissue-specific immune reactions in peripheral joints. Immunization with CII is associated with development of autoimmune arthritis in several species (6–8). Collagen-induced arthritis (CIA) shares many characteristics with human rheumatoid arthritis. As most extensively studied in mice, the development of CIA is strongly associated with certain MHC-II haplotypes (9, 10), indicating that the model is dependent on T cell recognition of a restricted set of CII peptides presented by appropriate MHC molecules (11). Indeed, peptides derived from the same region of CII (amino acid residues (aa) 256–270) are bound by both DR4 and Aα molecules (10, 12), whose expression is genetically associated with rheumatoid arthritis and CIA, respectively.

T cell recognition of proteolytically processed CII, however, does not meet all requirements for the development of arthritis. The induction of CIA, in fact, is critically dependent on immunization with CII in its native conformation, i.e. the intact triple-helical structure of collagen (6, 13). The conformation requirements, as well as the dependence on functional B cells (14, 15), indicate that autoreactive CII-specific B cells are crucial to the pathogenesis of CIA. In high responder mice such as DBA/1 (H2b), for example, there is apparent lack of negative selection of CII autoreactive B cells. As a consequence, immunization of DBA/1 mice with heterologous (rat) CII gives rise to early activation (day 5–9 after immunization) of IgG-secreting autoreactive B cells; these recognize a set of immunodominant native structures on the triple-helical moiety of the autologous CII molecule (14, 16). The autoantibodies are cross-reactive with CII from various species (i.e. human, chick, and bovine), but, within the same species, they do not cross-react with any of the systemically available collagens, for example type I (C1), despite a homology of 80% at the amino acid level, suggesting that CI-reactive B cells might have been negatively selected.

The pathogenic potential of CII-specific autoantibodies, in turn, is indicated by the fact that, after intraperitoneal injection of CII, monoclonal antibody; CII, collagen type II; C1, collagen type I; CIA, collagen-induced arthritis; HEB, human embryonic kidney; PBS, phosphate-buffered saline; RSA, bovine serum albumin; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s); D period, fibril period of collagen.
tion into syngeneic mice, they bind to articular cartilage (15) and induce synovial inflammation and even erosive arthritis (15, 17–19).

The aim of the present study was to characterize the dominant target structures of the B cell response on CII in the initial phase of CIA. For this purpose, epitope mapping was performed on a collection of B cell hybridomas isolated from lymph nodes and spleens of arthritis-prone DBA/1 mice on day 9–11 following immunization (14, 20). This collection of mAbs is well characterized in terms of binding to intact articular cartilage, arthritogenic potential, and representation of immunodominant epitopes (16, 17, 20). Since all antibodies bind in a conformation-dependent manner, attempts to map epitopes by synthetic peptides or purified enzymatic collagen fragments have achieved rather limited results (21). Therefore, we have established a method that allows the construction of recombinant homotrimeric collagen chimeras; this approach is based on the mutagenesis of the α1(X) collagen chain, allowing the presentation of epitopes in triple-helical conformation. This enables to characterize precisely the conformation-dependent binding of 36 mAbs and to identify the immunodominant B cell epitopes of CIA in DBA/1 mice, giving insight into the structural basis of autoimmune recognition of the most abundant collagen type in the joints.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—The hybridomas obtained during the primary response to immunization with CII were produced as follows. On day 0, DBA/1 mice (8–12 weeks old) were immunized in the right footpad with 50 μg of chicken or rat CII emulsified in complete Freund’s adjuvant (Difco, Detroit, MI); 9–11 days later, cells were obtained from the popliteal lymph nodes that drain the injection site and were fused with the SP2/0 myeloma cell line, obtaining the clones E1, E5, E10, E8, D7, D8, A12, C2, D3 (day 9 after immunization) and the clones F4, F10, C2P293–340, and H2O6 (day 11). For the characterization of the mAbs, some clones were derived from DBA/1 mice undergoing a secondary response; the immunization in this case proceeded as follows. On day 0, 50 μg of chicken or rat CII emulsified in complete Freund’s adjuvant were injected at the base of the tail; booster doses (50 μg of chicken or rat CII emulsified in IFA) were administered either intraperitoneally, intravenously, or subcutaneously. The spleens were removed and fused 21 days after obtaining the clones C1 and M2 76–332; or in the right footpad, in which case the popliteal lymph nodes were removed and fused 11 days after booster, obtaining the clones LN2 7–423. Each fusion was seeded on a 10-cm plate (1 × 10⁶ cells/plate) and grown overnight. Transfection was performed with 10 μg of the plasmid DNA construct by the calcium phosphate precipitation method. The cells were incubated for 24 h with a mixture of DNA and calcium phosphate in 10 ml of DMEM containing 10% FCS. This mixture was replaced by DMEM/F-12, 5% FCS; after 48 h, the selection was started by supplementation with 800 µg/ml G-418 (Life Technologies, Inc.). The medium was renewed every 2 days; the collection of supernatants was started when the G-418 resistant cells reached confluence. During harvesting, the transfected HEK 293 cells were kept in FCS-free DMEM/F-12 supplemented with ascorbate.

Immunoblots—Protein was precipitated from 1 ml of supernatant by the addition of 139 µl of Triton X-100 (1% in H₂O) and 250 µl of trichloroacetic acid (55% in H₂O). The precipitate was dissolved in 40 µl of sample buffer and immediately subjected to SDS-polyacrylamide gel electrophoresis using 10% gels. The separated proteins were subsequently electroblotted to a nitrocellulose sheet (Schleicher & Schuell, Dassel, Germany) using the semidyblot technique. The blots were blocked with 3% BSA in PBS and subsequently probed with a mAb X53 raised against recombinant collagen X (Ref. 29; generous gift of Dr. I. Girkontaite, Institute of Experimental Medicine, Erlangen, Germany). Specific binding was detected using horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dianova, Hamburg, Germany) and ECL detection (Amersham, Braunschweig, Germany).

ELISA—Serum-free cell culture supernatants from transfected HEK 293 cells were directly used for coating of ELISA plates (Nunc, Wiesbaden, Germany). Media from non-transfected HEK 293 cells, as well as supernatants from transfected HEK 293 cells propagated in Bluescript (New England Biolabs, Beverly, MA) and a syngeneic control IgG2a mAb (F3) were purified, biotinylated, dialyzed against phosphate-buffered saline (PBS), and filtered. The specific binding was detected using horseradish peroxidase-conjugated antibody to mouse IgG (Dianova), biotinylated secondary antibody, and avidin-biotin-peroxidase complexes (25). The reaction was developed with 3-amin-9-ethylcarbazol containing H₂O₂. All sections were counterstained with Mayer’s hematoxylin.

Vector Construction—The vectors for the expression of the chimeric collagen molecules were based on a human collagen X cDNA clone (26) propagated in Bluescript (New England Biolabs, Beverly, MA) and a human CII cDNA clone (kindly provided by Dr. S. W. Li and Prof. D. J. Prockop, Jefferson University, Philadelphia, PA). The CII cDNA was used as template for the PCR amplification of parts of the CII sequence. Specific restriction sites (XhoI/NotI, PstI, and BamHI), which allowed cloning of these fragments, were introduced via the PCR primers. Very short CII fragments (30–50 bp) were directly generated from annealed complementary synthetic oligonucleotides, which were subsequently filled with nucleotides by Klenow DNA polymerase (27). The CII fragments and the collagen X cDNA clone were digested with the respective restriction enzymes (NcoI/BamHI or PstI/BamHI), and the corresponding DNA fragments were purified by agarose electrophoresis. The CII fragments were ligated in frame into the collagen X cDNA, resulting in the corresponding chimeric CX-CII constructs in Bluescript. For the expression in eukaryotic cells, these chimeric CX-CII constructs were cloned in the pRCMV vector (Promega, Madison, WI). Prior to the transfection procedure, the chimeric constructs were controlled by in vitro translation and DNA sequencing (28).

HEK 293 Cell Culturing and Transfection—HEK 293 cells, maintained in Dulbecco’s modified Eagle’s medium (DMEM/F-12), were seeded on a 10-cm plate (1 × 10⁶ cells/plate) and grown overnight. Transfection was performed with 10 μg of the plasmid DNA construct by the calcium phosphate precipitation method. The cells were incubated for 24 h with a mixture of DNA and calcium phosphate in 10 ml of DMEM containing 10% FCS. This mixture was replaced by DMEM/F-12, 5% FCS; after 48 h, the selection was started by supplementation with 800 µg/ml G-418 (Life Technologies, Inc.). The medium was renewed every 2 days; the collection of supernatants was started when the G-418 resistant cells reached confluence. During harvesting, the transfected HEK 293 cells were kept in FCS-free DMEM/F-12 supplemented with ascorbate.
Conformation-dependent B Cell Epitopes in CIA

RESULTS

Antibody Specificity for the Native Conformation of Collagen II

All mAbs bound in a conformation-dependent manner to CII, i.e., they required the triple helix of the epitope, as shown in a representative ELISA with native and heat-denatured CII for mAb D3 (Fig. 1B). The binding ability of mAb D3 was lost after denaturation. The control antibodies, i.e., the isotype-matched mAb B1, recognizing a linear collagen epitope (Fig. 1B), and a CII-specific polyclonal rabbit antibody, did bind to CII under all conditions tested (results not shown). Heat-denatured CII was exposed to antibodies at 37 °C, and not at room temperature, to prevent refolding of the collagen triple helix.

Antibody Specificity for the Native Conformation of Collagen II

The conservation of triple helicity of recombinant collagen molecules was studied in the presence of antibodies, as well as a subunit of its heterotrimeric structure (35). A representative example (mAb F4) for antibody specificity is shown in Fig. 1A.

Antibody Binding—The intraperitoneal injection of the biotinylated mAbs E8, D3, and C2, but not that of the control mAb F3 (which does not bind to CII), led to antibody binding to the articular cartilage. In the case of the mAb D3, the binding could be considered specific because blocked by an anti-idiotypic antiserum in vivo (36). Single staining with avidin-peroxidase confirmed the binding of the biotin-conjugated anti-CII mAbs, leaving the synovia unstained, as expected (data not shown).

Characterization of the Recombinant Chimeric Collagen Molecules

For precise mapping of the triple-helical epitopes, chimeric collagen constructs were made using the frame of the human full-length a1(X) cDNA to insert different fragments of the human a1(II) cDNA. For this purpose, a 295-bp-long a1(X) fragment flanked by the unique restriction sites PstI and BamHI, or a 338-bp-long cassette generated by restriction with NcoI and BamHI, was replaced by PCR-amplified a1(II) inserts of varying length. Initially, constructs were generated that contained larger a1(II) inserts (up to 900 bp), to cover the entire length of the CII molecule with a few recombinant collagen molecules. Subsequently, a total of 130 recombinant chimeras were constructed to localize the B cell epitopes precisely on different recombinant chimeric collagen molecules containing a1(II) inserts with only small sequence overlaps. To maintain stable triple helices, the recombinant collagen chimeras were constructed without interfering with the Gly-X-Y sequence. Collagen X was chosen, since it is a homotrimeric molecule and none of the mAbs cross-reacted with this collagen type (Fig. 1A). Recombinant chimeric collagen XIX molecules were produced by transfection of HEK 293 cells with the chimeric constructs and expression under the cytomegalovirus promoter. The Western blot in Fig. 3, developed with the collagen X-specific mAb X53, demonstrates the differences in electrophoretic mobility of the recombinant collagen X chimeras depending on the varying length of a1(II) inserts (bp positions as in Ref 37). The conservation of triple helicity of recombinant collagen was controlled by resistance to trypsin/chymotrypsin digestion up to a melting temperature of ~43 °C (results not shown).
Characterization of the B Cell Epitopes

One hundred and thirty different chimeric collagen constructs were expressed in HEK 293 cells. Cell culture medium was coated to ELISA plates and coating efficiency controlled by a specific anti-CX mAb prior to the mAb testing. From the original 45 hybridomas, 9 mAbs showed high background reactivity and were not further investigated. For the remaining 36 mAbs, epitopes were mapped by constructing a series of overlapping fragments. After testing these fragments by ELISA, the mAb binding sites were thereby narrowed down to stretches of 5–14 aa within the CII triple helix. Table I summarizes the binding properties of six representative mAbs to a selected number of chimeras.

The minimal CII-specific sequences required for recognition are highlighted in Table I. These binding regions for representative mAbs were defined as epitopes C1III, J1, D3, E/F10 I, E/F10II, and F4. Remarkably, the epitopes E/F10 I and E/F10II differed only by 1 aa. An even subtler microdiversity in antibody recognition could be detected in the region of the C1III epitope, as revealed by the reactivity of the mAbs C1, LN 2.7, and CB300 (Fig. 4). Three overlapping, but nevertheless clearly distinct epitopes (C1I, C1II, and C1III) were identified between bp positions 1623 and 1655 (aa 359–369).

The extensive mapping strategy using the entire set of recombinant chimeras was applied on each of the 36 mAbs; a summary of the results is given in Fig. 6 and Table II, which also contains the information about the V-gene usage of the CII-specific B cells (20). From the data of Table II, it is evident that the epitopes presented in Table I and Fig. 4 are indeed dominant targets of the B cell response, since each was recognized by more than one mAb.

The binding regions of mAbs recognized by only one antibody (Table II) were not systematically mapped; however, the localization of their epitopes was confined to regions shorter than 50 aa with a few exceptions (M2 136, M2 191, and LN2; Ref. 15). These singly recognized epitopes turned out to spread along the entire triple helix and could be localized on all cyanogen bromide fragments (CB) of CII.

The specificities of the remaining 27 mAbs were clustered to...
The N- and C-terminal ends of the epitopes (shaded areas) were identified by ELISA testing of six representative CII-specific mAbs against a selected number of chimeric collagen constructs of the 130 originally obtained. These chimeric constructs differed by their variable $\alpha_1$(II) inserts (indicated by the bp position in the $\alpha_1$(II) cDNA) into the constant frame of the $\alpha_1$(X). The mAb X53, a collagen X-specific antibody, was used to control for the expression of recombinant collagen chimeras. Recombinant CII ($\alpha_1$(II) 229-3833) controlled for the reactivity of the CII-specific mAbs (D8, M2.76, D3, F10, E10, and F4) and the specificity of mAb X53. The epitopes were designated C1III, J1, D3, E/F10I, F4.

### Table I

Mapping of CII-specific B cell epitopes as revealed by the use of recombinant chimeric collagen molecules

| CONSTRUCTS | MONOCLONAL ANTIBODIES |
|------------|------------------------|
| $\alpha_1$ (II) (bp) | D8 | M2 76 | D3 | F10 | E10 | F4 | control : X53 |
| 229-3833 | + | + | + | + | + | + | - |
| 1614-2375 | + | - | - | - | - | + | + |
| 1623-1737 | + | - | - | - | - | + | + |
| 1629-1737 | - | - | - | - | - | - | + |
| 1092-1649 | - | - | - | + | + | + | - |
| 1092-1655 | - | - | - | + | + | + | - |
| 1623-1655 | + | - | - | - | - | + | + |
| 2199-2240 | - | + | - | - | - | - | + |
| 2208-2249 | - | - | - | - | - | - | + |
| 2199-2240 | - | + | - | - | - | - | + |
| 2607-3230 | - | - | + | + | + | + | - |
| 2610-2987 | - | - | - | + | + | + | - |
| 2586-2618 | - | - | - | - | - | - | + |
| 2388-2639 | - | - | - | - | - | - | + |
| 2607-2645 | - | - | + | - | - | - | - |
| 2586-2642 | - | - | + | - | - | - | + |
| 2847-2987 | - | - | - | + | + | - | - |
| 2874-2987 | - | - | - | + | + | - | + |
| 2877-2987 | - | - | - | - | + | - | + |
| 2880-3032 | - | - | + | - | - | - | - |
| 2595-2888 | - | - | + | - | - | - | - |
| 2595-2894 | - | - | + | - | - | - | - |
| 2595-2897 | - | - | + | + | + | - | - |
| 2874-2997 | - | - | - | + | + | - | + |
| 3315-3545 | - | - | - | - | - | - | - |
| 3342-3374 | - | - | - | - | - | - | - |
| 3315-3347 | - | - | - | - | - | - | - |
| 3324-3356 | - | - | - | - | - | - | - |

### Epitope Borders

| (bp) | 1623 | 2199 | 2607 | 2874 | 2877 | 3342 |
|------|------|------|------|------|------|------|
| 1655 | 2240 | 2642 | 2897 | 2897 | 3386 |

| (aa) | 359 - 369 | 551 - 564 | 687 - 698 | 776 - 783 | 777 - 783 | 932 - 936 |

| EPI TOPE | C1 III | J1 | D3 | E/F10 | E/F10 | F4 |
|----------|--------|----|----|------|------|----|
eight major epitopes (Table II) and localized in five distinct regions (C1I-11, J1, D3, E/F10-I1, and F4) on the CB11, CB10, and CB9. These immunodominant regions were not only characterized by binding of several antibodies to one epitope, but also by clustering of overlapping, yet distinct epitopes. This was most impressive in the dominant region within the CB11 fragment (C1I-11). Within this 11 aa stretch (aa 359–369), three different epitopes were recognized by as many as 15 mAbs, i.e. 41.7% of the entire collection to a 11-aa-long stretch. No significant differences in absorbance were detectable with the CX specific mAb X53 (data not shown), thereby excluding any bias by a variation in the coating efficiency between the chimeric collagens.

The mapping and characterization of the arthritic epitopes of CII, necessary to understand the link between B cell immunity and disease, has revealed so far a number of linear epitopes covering the entire sequence of the immunodominant CNBr-fragment 11 (α1(II)/CB11) (41, 42). However, since CII epitopes require a triple-helical conformation to be arthritogenic, and since they are difficult to reveal due to the limited accessibility of rotary shadowing (± 30 aa) (21), a novel strategy was developed in the present study, based on the insertion of a series of CII cassettes into triple-helical collagen X. The use of a eukaryotic expression system resulted in successful presentation of chimeric collagens in triple-helical conformation. In addition to proving relevant for unraveling B cell epitopes, the present method can find widespread applicability for the elucidation of other conformation-dependent ligand interactions with extracellular matrix components, for example that of collagen with integrin (40), decorin (43), or fibromodulin (44).

The set of mAbs investigated in the present study is well characterized for their pathogenic importance, i.e. for their capacity of inducing synovial inflammation; indeed, CIA is reversed by administration of an anti-idiotypic mAb (C1C3) detecting a cross-reactive idiotype shared by mAbs C1, C2, and E10 (45). Competitive ELISA assays document as well that a substantial part of antibodies in the sera of arthritic mice binds to the same epitopes (46). While single B cell epitopes proved to spread along the entire triple helix of CII, the statistical analysis uncovered binding clusters in five distinct domains. Most remarkable is the binding of 41.7% of the entire mAb collection to a 11-aa-long stretch within the CB11 (ARGLTGRPGDA). Within this major region, there was clear microheterogeneity, since the fine mapping for the minimal epitope requirements revealed not less than three distinct epitopes of varying length (5–11 aa) starting at a con-
A

| CB  | mAb | SEQUENCE | position in α1(II) triple helix |
|-----|-----|----------|-------------------------------|
| CB11| C1  | ARGLT    | aa 359-363                    |
| LN2.7|     | ARGLT    | aa 359-366                    |
| D8  |     | ARGLT    | aa 359-369                    |
| M276|     | ARGAT    | aa 551-564                    |
| D3  |     | ARGAT    | aa 687-698                    |
| E10 |     | ARGTV    | aa 777-783                    |
| F10 |     | ARGTV    | aa 776-783                    |
| CB9 | F4  | ARGFT    | aa 932-936                    |

CONSENSUS

R G hydrophobic

B

EPITOPE E/F 10 II:

Comparison between Collagen I and II

Primary sequence:

α1(I) AQQRGTV

α1(II) AQQRGTV

α2(I) LGAPQIL

Triple helical assembly

Collagen II: [α1(II)]₃

Collagen I: [α1(II)]₂α2(I)

Fig. 5. A, amino acid sequences of the immunodominant B cell epitopes on CII. Eight distinct epitopes were identified on the CNBr-fragments CB11, CB10, and CB9. Microdiversity was found in the regions of aa 359–369 (C1, C11, C111) and aa 776–783 (E/F10, E/F100). All epitopes contained a consensus motif R G hydrophobic (highlighted). B, schematic representation of the mAb epitope E/F10 on the surface of the triple-helical α1(II) molecule. In the upper part, the sequence of α1(II) is compared with the respective sequences of the heterotrimeric collagen I, to which the corresponding mAb E10 does not bind. In the lower part, the side chains of the aa residues are depicted on the cylindrical surface of the collagen molecules in triple-helical assembly, based on the model by Eble et al. (40). The open circles represent the glycine residues that remain hidden in the cylinder axis. This presentation of the epitope localization demonstrates how the presence of the α2 chain interferes with the sterical conformation of the epitopes on the cylindrical surface of the triple helix.
Conformation-dependent B Cell Epitopes in CIA

Fig. 6. Distribution of antibody binding along the entire triple helix of monomeric CII. The position of the antibody epitopes with the entire sequence of the CII triple helix is shown on the x-axis. In addition to the amino acid numbering, the borders of cyanogen bromide fragments (CB) on CII are indicated to facilitate localization of the epitopes. On the Y-axis is shown the number of mAbs binding to a particular site along the CII molecule. The bold bars represent antibodies with exactly determined epitopes (length < 14 aa residues). These mAbs bind to determinants that are recognized by more than one mAb and represent the eight major epitopes in five immunodominant domains regions (designated C1ІІІ, J1, D3, E/F10ІІІ, and F4) since a region on CB10 (E/F10ІІІ, aa 776–783) and another on CB11 (C1ІІІ, aa 357–368) contain overlapping epitopes. The C1ІІІ domain is the most frequently recognized region. Fifteen mAbs (41.7% of the total) bind to this site.

Served N terminus. A similar microheterogeneity distinguished an epitope localized in the CNBr-fragment 10 (αCIІІ) CB10), close to the collagenase cleavage site. The present results confirm therefore that immunodominance of CII is limited to a few discrete domains on the 1014-aa-long triple-helical moiety. Notably, the most frequently recognized epitope, localized on the CNBr fragment αII(II) CB11, was previously identified as the predominant target of B cell response in DBA/1 mice (13, 16). Accordingly, only immunization with renatured CIICB11, but not with other CNBr fragments, induces experimental arthritis (13). Hence, the CIICB11 contains structures of crucial importance for the induction of autoimmune arthritis in DBA/1 mice; the present data indicate that the triple helix between aa 359 and 369 is one of these structures, namely the immunodominant B cell epitope. It is likewise remarkable that the αII(II) CB11 also harbors the dominant T cell determinants (aa 256–270) for recognition of CII (11, 47).

Although the five dominant B cell epitopes are spread over a considerable distance on the monomeric CII molecule, their distribution is unlikely to be random. In cartilage, CII molecules assemble in a multimeric, quarter-staggered fibrillar array. On the basis of the current model for packing of molecules in native fibrils (48, 49), the calculation of the position of the single epitopes in the quaternary structure of collagen revealed a spatially ordered distribution of the immunodominant domains (Fig. 7A). In this multimeric structure, the E/F10 and J1 epitopes, as well as the D3 and F4 epitopes, coalesce in clusters within two distinct narrow regions (corresponding to a length of < 20 aa) on neighboring triple helices. Thus, the repetitive D period of 66 nm length of the collagen microfibril is likely subdivided into three segments, proportional in size at a ratio of approximately 2:1:2 by the three loci of B cell epitope clusters (E/F10/J1, C1ІІІ, and D3/F4; Fig. 7A).

In intact cartilage, the microfibril structure is even more complex, due to the heterotypic interactions of CII with other extracellular matrix molecules, e.g. collagen IX (50, 51), collagen XI (52), decorin (43), or fibromodulin (44). Such interactions determine structural constraints on the accessibility of collagen for antibody recognition; the spatially ordered distribution of immunodominant B cell epitopes may thus result from such constraints. In the heterotypic fibrils, the collagen surface is largely masked by other extracellular matrix molecules. The immunodominant epitopes are, however, apparently localized in less densely coated regions of the CII molecules and may be thus accessible to the early antibody response characteristic of the initial phase of CIA.

Peculiarities of B cell epitope localization in the heterotypic fibril emerge when the relative molecular arrangements of collagen IX and XI to CII are reconstructed on the basis of a model that takes into account the precise localizations of intermolecular cross-links and the constant dimensions of the rigid triple-helical molecules (50–52). Although the contribution of decorin and fibromodulin cannot be estimated due to the lack of information on their arrangement, all B cell epitopes appear to localize either within the gap region (C1ІІІ), less densely packed than the overlap region, or at the borders of the gap region (E/F10ІІІ/J1 and D3/F4) (Fig. 7A). In the heterotypic arrangement, the telopeptide regions of collagen II and XI, as well as three of four non-collagenous domains of collagen IX, form clusters to the same regions of the D period on CII to which the dominant B cell epitopes have been mapped (Fig. 7B). The relative flexibility of non-collagenous domains of collagen may thus uncover triple-helical domains in their close vicinity, especially upon mechanical stress in the fibrillar network during movement.

The in vivo binding of the mAbs to normal cartilage did not require further unmasking procedures (Ref. 15 and Fig. 2A); thus, the B cell epitopes appear more easily accessible than other CII regions. In the case of the two clusters that reside close to the gap region of the collagen fibril, the triple helices in their immediate vicinity are sufficiently exposed to permit enzymatic attack during normal matrix metabolism. The E/F10ІІІ epitope maps in fact to position aa 776, which is directly adjacent to the unique collagen cleavage site at aa 775 (38, 39). The F4 epitope, in turn, starts at aa 932 in the immediate vicinity of the lysine residue in position 930, which is one of the two hydroxylysines in CII triple helices (aa 87 and 930) involved in intermolecular cross-linking (49). The aldehyde-derived cross-links require the precedent action of the enzyme lysyl oxidase (53) on adjacent (hydroxy)lysines of the neighboring telopeptide regions (as indicated in Fig. 7B), indicating that this site is accessible in the native fibril. Thus, the colocalization of immunodominant B cell epitopes with sites of selective enzymatic modification suggests a structural peculiarity that may permit distinction from the repetitive Gly-X-Y surrounding. The immunodominant B cell epitopes share a consensus motif RG hydrophobic. This conserved sequence, however, does not define all requirements for specific binding, since there is
no cross-reactivity between mAbs that have been mapped to different immunodominant regions. Thus, the aa residues flanking the consensus are also important for specific interaction with the complementarity determining regions of the mAbs. The importance of the flanking sequences may be due to the homotrimeric assembly of CII (as depicted in Fig. 5B), which leads to considerable changes of the triple-helical surface as a result of only one aa exchange in the primary sequence. Antibody recognition of microdiverse structures on the cylindrical collagen surface may therefore circumvent cross-reactivity among different members of the highly homologous collagen family.

In this study, the majority of B cell hybridomas was derived during the course of the primary response to CII. V-gene analyses of the hybridomas revealed that the antibodies are germ-line-encoded and exhibit a recurrent usage of single genes from the VHJ558, VH24, and VK21 families (20). Moreover, there was even sharing of V-genes, i.e. the same V-gene was found in hybridomas binding to different epitopes on the same antigen (VK21C is used for recognition of C1I, D3, and F4; Table II). This heavily biased V-gene combination (Table II and 20) indicates that conserved germine structures in the V-regions are important for CII binding (22). Thus, the recurrent V-gene usage may reflect selection of B cell populations based on antigen-specific structural constraints. It should be noted that immunization of DBA/1 mice with CII induces an IgG-switched

| mAB  | mouse | V_H | V_L | Ig ISOTYPE | EPITOPE | EPITOPE LOCALIZATION |
|------|-------|-----|-----|-----------|---------|---------------------|
| LN2 49 | b     |     |     | nd        | CB12    | 94 - 123            |
| CB 286 | g     |     |     | G2b      | CB12    | 209 - 219          |
| M2 136 | a     | J558 |     | G1       | CB11    | 221 - 336          |
| H206   | h     |     |     | G2a      | CB11    | 347 - 399          |
| C1 268 | i     | J558 |     | G1       | CB11    | 359 - 363          |
| CB 189 | d     |     |     | nd       | CI'     | CB11    | 359 - 363          |
| E 5    | m     | Q52  | nd  | G1       | CI'     | CB11    | 359 - 363          |
| D 7    | s     |     |     | G2a      | CI'     | CB11    | 359 - 363          |
| LN2 7  | b     | J558 |     | G2b      | CI''    | CB11    | 359 - 366          |
| CB 239 | i     | J558 |     | G2a      | CI''    | CB11    | 359 - 366          |
| A 12   | r     |     |     | G2b      | CI''    | CB11    | 359 - 366          |
| D8 300 | g     | J558 |     | G3       | CI''''  | CB11    | 359 - 369          |
| CB 313 | g     | J558 |     | G2b      | CI''''  | CB11    | 359 - 369          |
| M2 87  | a     | J558 |     | G2a      | CI''''  | CB11    | 359 - 369          |
| M2 88  | a     | J558 |     | G2a      | CI''''  | CB11    | 359 - 369          |
| M2 328 | c     |     |     | nd       | CB11    | 377 - 399          |
| M2 191 | a     | S107 |     | G2a      | CB8/CB10| 467 - 585          |
| M2 76  | a     | J558 |     | G1       | J1      | CB10    | 551 - 564          |
| M2 139 | a     | J558 |     | G2b      | J1      | CB10    | 551 - 564          |
| M2 332 | c     | J558 |     | G2b      | J1      | CB10    | 551 - 564          |
| LN2 48 | p     |     |     | nd       | J1      | CB10    | 551 - 564          |
| D3 173 | f     | X24  |     | G2a      | D3      | CB10    | 687 - 698          |
| C2 417 | f     | X24  |     | G2a      | D3      | CB10    | 687 - 698          |
| LN2 15 | b     | 36-60|     | G1       | F4      | CB9     | 932 - 936          |
| FN 423 | f     | 36-60|     | G2a      | F4      | CB9     | 932 - 936          |
| M2 145 | a     |     |     | nd       | CB9     | 971 - 999          |
response which is not preceded by a typical IgM surge (20); this constellation, reminiscent of a recall rather than a primary response, suggests that selection of CII-autoreactive B cells might occur in the naive animal prior to immunization. The identified triple-helical consensus motif may thus represent a conserved repetitive recognition structure for B cells; this structure may be critically involved in V-gene selection for autoantibodies relevant to the pathogenesis of CIA.

The spatially ordered distribution of the dominant epitopes containing the R G hydrophobic motif is reminiscent of the B cell epitope organization on paracrystalline surface structures of pathogens. In the case of the virus envelope glycoprotein VSV-G, for example, the contact of mature B cells with antigen in an optimal rigid repetitive epitope organization (in the absence of cognate T cell help) does not necessarily lead to anergy or apoptosis, but rather to activation of B cells via optimal Ig receptor cross-linking (54). It is therefore an intriguing possibility that the early occurrence in arthritis-prone DBA/1 mice of V-gene-selected B cells secreting anti-CII IgG are connected to the repetitiveness of determinants in discrete accessible sites of collagen fibrils in cartilage; this may result in positive selection rather than deletion of autoreactive B cells. B cells, in turn, could be exposed to CII in the bone marrow during cartilage resorption in enchondral bone formation; once left the bone marrow, these B cells may encounter cognate T cell help in the periphery during exposure to antigen. This sequence of events may shape a pre-activated pool of clonally selected CII-specific B cells, present already in naive animals. Upon various provocations, such as immunization with CII, this preshaped pool could be recruited in arthritogenic immune responses; this may occur on one hand by secretion of anti-CII IgG, and on the other hand by lowering the threshold for engagement of autoreactive T cells, due to the efficiency of activated B cells in antigen presentation (55, 56). Although it cannot be formally excluded that the selection process of CII specific B cells is initiated by the immunization and governed by requirements for T cell help, we suggest a preimmune impact that is biased by the accessibility of CII determinants in the intact cartilage tissue. This selection may explain why autoantibodies bind to
intact cartilage in vivo, and challenges the concept that CII is an immunoprivileged self protein.

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