VH3-21 B Cells Escape from a State of Tolerance in Rheumatoid Arthritis and Secrete Rheumatoid Factor

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ABSTRACT

Background: Rheumatoid factor (RF) is a characteristic but not pathognomic feature in patients with rheumatoid arthritis (RA). It is unknown whether the repertoire of immunoglobulin genes utilized by RF^+ B cells of RA patients is unique and whether RF^+ B cells in normal individuals are silenced or deleted.

Materials and Methods: Clonal B cell populations were established from the peripheral blood of normal donors (127 B cell clones), RA patients (113 RF^- and 60 RF^+ B cell clones) and patients with primary Sjögren's syndrome (82 RF^- and 47 RF^+ B cell clones) by coculturing with anti-CD3–stimulated T helper cell clones. The cross-reactivity pattern of antibodies secreted by the B cell clones was determined by ELISA on a panel of antigens. The molecular structure of the IgM heavy chains was characterized by VH family–specific RT-PCR and sequencing. VH elements which correlated with RF specificity were identified. The responsiveness of B cells expressing these VH elements to T helper cell signals was compared in normal individuals and RA patients.

Results: The majority of RF^+ B cells were monospecific when specificity was tested on five antigens. RF^+ B cells expressed a significantly different repertoire of VH gene segments than RF^- B cells. In particular, the VH3 gene segment V3-21 was not detected in B cell clones from normals but was the most frequent VH element in RF^+ B cell clones from RA patients. Most of the V3-21 sequences were in germline configuration. The correlation between RF specificity and V3-21 gene segment usage was maintained in patients with Sjögren's syndrome. V3-21 transcripts were present in peripheral blood B cells from normal individuals. $VH3\text{-}21^+$ B cells from RA patients but not from normal donors were responsive to preactivated T helper cells. Stimulation with a bacterial superantigen could overcome the nonresponsiveness of $V3\text{-}21^+$ B cells in normal donors and induce the secretion of RF.

Conclusions: RF production is correlated with the usage of the V3-21 gene segment in two distinct RF^+ diseases. In patients with these diseases, $V3\text{-}21^+$ B cells secrete antibodies with RF activity in response to activated T helper cells. $V3\text{-}21^+$ B cells remain in a state of nonresponsiveness in normal individuals that can be broken by superantigen stimulation. The germline configuration of $VH3\text{-}21^+$ RF^+ immunoglobulins in RA patients suggests that the loss of tolerance is not an antigen-driven process.

INTRODUCTION

Rheumatoid factor (RF) recognizing the Fc portion of IgG molecules makes up the majority of the autoantibodies produced by patients with rheumatoid arthritis (RA) (1–3). Although most frequently found in RA, production of RF is neither consistently associated with RA nor is it specific for RA. In particular, patients with primary Sjögren's syndrome (pSS), scleroderma, and mixed connective tissue disease can have circulating antibodies that react with IgG Fc epitopes (1). RF can also be produced by normal individuals, and an increase in titers of IgM RF is a phenomenon frequently encountered in patients with ongoing chronic immune responses such as bacterial endocarditis or transplantation (4,5). For several decades, RF has been considered to be involved in the pathogenetic events and the inflammatory response of RA. The observation that RF undergoes affinity maturation in RA has supported the model that it is the products of an antigen-driven response, possibly initiated or maintained by a disease causative antigen.

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More recently, a different view of RF and RF^+ B cells has emerged (6). Evidence has been presented that RF^+ B cells serve important physiological functions. Their ability to bind IgG-containing immunocomplexes may give them a clearing function and enable them to bind and present antigenic peptides from antigens bound in immune complexes (7). Generally, B cells are able to take up their specific antigen at much lower antigen concentrations than other professional APC (8,9). Thus, RF^+ B cells could represent a population of APC that express a wide variety of antigenic peptides in their HLA molecules reflecting upon the antigens trapped in IgG complexes.

Provided that RF^+ B cells are present in the normal immune repertoire and serve an important physiological function as APC for antigens present in the form of immune complexes with IgG, these cells are obviously not clonally deleted (10). It is unclear how RF^+ B cells in normal individuals are maintained in a functional state which allows them to bind IgG antigen complexes through their membrane-anchored immunoglobulin but prevents secretion of this immunoglobulin. By using a transgenic mouse model for human IgM RF, Tighe et al. have provided in vivo evidence for the model that RF^+ B cells are important in antigen presentation (11). Interestingly, despite functional competence of RF^+ B cells in these mice, antibodies with anti-Fc reactivity were not secreted. Secretion of RF could be induced by introducing a gene of the MRL/lpr background, and the authors have hypothesized that similar mechanisms may underlie the secretion of RF in the absence of a known antigenic stimulus in diseases such as RA and pSS. As an alternative model, it has been suggested that the production of an RF autoantibody is closely linked to affinity maturation. Somatic mutation and class switching are infrequent events in RF produced by normal individuals (12). In contrast, RA patients may lack the ability to control affinity maturation of RF-producing B cells resulting in the selection of somatically mutated, high affinity RF.

The analysis of the regulation of RF-producing B cells in patients with RA has been hampered due to the fact that RF^+ B cells in RA patients cannot be easily identified. Here, we report that IgM RF^+ B cells in patients are characterized by a unique immunoglobulin gene segment usage providing a molecular marker for this B cell subset. By studying the IgM heavy chain of B cell clones established from peripheral blood of RA patients and normal individuals, we have found a correlation between the expression of the VH gene segment V3-21 and RF specificity. $V3\text{-}21^+$ B cells were present in the repertoire of normal individuals, but they did not proliferate to signals provided by anti-CD3 activated T helper cells. In contrast, $V3\text{-}21^+$ B cell clones could be established from the peripheral blood of patients with RA. These B cells preferentially secreted monospecific RF, however, the IgM heavy chain did not show any evidence for somatic mutation. Similar results were obtained from patients with pSS, another disease which is characterized by the production of RF. Our data suggest that patients with RA and pSS share a regulatory defect in controlling the activation of these RF-producing B cells, which remain non-secreting B cells in normal individuals.

MATERIALS AND METHODS

Study Population

Thirteen patients with seropositive RA, 13 normal individuals, and two patients with pSS were studied. All patients with RA fulfilled the American College of Rheumatology diagnostic criteria (13). The normal donors did not have a personal or family history of an inflammatory rheumatic disease, and all of them tested negative for RF. The two patients with pSS fulfilled five classification criteria proposed for the diagnosis of pSS (14). They were positive for RF, anti-SS-A, and anti-SS-B antibodies but lacked clinical features suggestive of RA, SLE, polymyositis, or scleroderma. In particular, they did not have erosive joint disease.

Generation of B Cell Clones

B cell clones were established by coculturing a limited number of B cells (3–5 cells/well) with anti-CD3 activated clonal T cells as recently described (15). In brief, B cells were purified from PBMC by treatment with 50 mM L-leucine methyl ester HCl (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 45 min at room temperature and subsequent rosetting of the surviving cell population with AET-treated sheep red blood cells (16). Nonrosetting cells were highly enriched in B cells and contained between 75 and 90% of $CD19^+$ lymphocytes as demonstrated by FACS analysis. The T cell clone LAB1-10, used in this study, is alloreactive and recognizes HLA-

DRB1*0404$^+$ stimulator cells, as previously described. None of the B cell donors expressed an allogeneic specificity recognized by LAB1-10 (data not shown). B cell clones were established by coculturing 1×10^5 cloned T cells (LAB1-10) with three to five B cells per well in 96-well flat-bottom tissue culture plates coated with anti-CD3 mAb. After 11 days, microcultures were fed with 20 U/ml rIL-2 (Cetus). In selected experiments, B cell lines were established by coculturing 5×10^3 B cells with 1×10^5 LAB1-10 in the presence of immobilized anti-CD3 and 1 μg/ml staphylococcal enterotoxin D (SE D) (Toxin Technology, Sarasota, FL, U.S.A.), respectively.

Antibody Specificity Testing

Culture supernatants were collected after 18–21 days of culture and total IgM and antibody specificity were determined in an ELISA assay. To determine IgM, a sheep anti-human immunoglobulin (Sigma) was coated to maxisorb plates (Nunc Inc., Naperville, IL, U.S.A.). After blocking, plates were incubated with undiluted supernatants and then incubated with alkaline phosphatase coupled goat anti-human IgM (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). To identify RF-producing cultures and to determine cross-reactivity patterns, plates were coated with human Ig-Fc fragments (Calbiochem-Behring Corp., San Diego, CA, U.S.A.), actin, keratin, single stranded DNA, thyroglobulin (Sigma), and tetanus toxoid (Behring Werke, Marburg, Germany), respectively. Subsequently, the plates were incubated with the supernatants. Alkaline phosphatase coupled goat anti-human κ- and λ-chain–specific anti-sera were used as developing antibodies. Serial dilutions of purified antibodies or sera with known antibody titers were included as positive controls. All assays were developed using p-nitrophenol phosphate in 10 mM diethanolamine buffer. Supernatants from cultures without any B cells were used as negative controls. Microcultures with signal intensities of at least three standard deviations larger than the signals from the control cultures were identified as positive.

Molecular Characterization of IgM Heavy Chains

Microcultures positive for IgM were identified and the cloning efficiency was determined. Cells from microcultures which were likely to be clonal were harvested, washed with phosphate-buffered saline (PBS), and lysed with 4 μl of NP40 lysis buffer (1% NP40, 10 mM Tris HCl, 10 mM NaCl, 3 mM $MgCl_2$, pH 8.0, and 0.5 U RNAse inhibitor). cDNA was synthesized in a total volume of 20 μl by incubation with 0.5 U AMV reverse transcriptase for 2 hr at 37°C. cDNA was amplified by polymerase chain reaction (PCR) using two mixtures of two VH-specific primers (GGTGCAGCTGGTGCAGTCTGG and GGTCAA CTTAAGGGAGTCTGG, GGACTGGTGAAGCCT TCGG and GGTACAGCTGCAGCAGTCAGG) and a Cμ primer (GGAATTCTCACAGGAGACGAG). These primer combinations amplified all VH gene families as previously shown (17). The amplified products were reamplified using internal VH family–specific primers (VH1, GTGAAGGTTTCCTG CAAGGCT; VH2, CTGACACTGAACTGCACCTTC; VH3, CTGAGACTCTCCTGTGCAGCC; VH4, CTG TCCCTCACCTGCGCTGTC; VH5, GGGGAGTCTCT GAGGATCTCC; VH6, CTCTCACTCACCTGTGCCA TC) and an internal Cμ primer (TGGGGCGGATG CACTCCC). Amplified products were separated on agarose gels and identified by ethidium bromide staining.

VH3$^+$ sequences were amplified using the Cμ primer and a VH3 family–specific primer, either one of which was attached to a T7 promoter. The amplified products were transcribed using a T7 RNA polymerase, and the transcripts were sequenced by transcriptase-mediated dideoxy-sequencing using a ^{32}P-endlabeled internal Cμ and a VH3 primer, respectively, as a reverse transcriptase primer as recently described (17,18). Sequences were read in both directions. All sequence reactions yielded unequivocal sequences, again supporting the concept that the microcultures were truly clonal. B cell clones were assigned to published sequences of the VH3, DH, and JH germline genes (19–27). DH segments were assigned using the criteria recently described (28).

Oligonucleotide Hybridization Assays with V3-21– and V3-23–Specific Primers

The frequency of V3-21 and V3-23 transcripts was semiquantified in freshly separated PBMC from 10 patients with RA and 10 normal controls and in B cell lines cocultured with T cell clones stimulated with either immobilized anti-CD3 or the SE D by using an oligonucleotide hybridization assay as recently described (29). cDNA was amplified with VH3- and IgM-specific primers.

TABLE 1. Frequencies of RF-secreting B cells in peripheral blood B cells from normal controls and patients with rheumatoid arthritis (RA) and primary Sjögren's syndrome (pSS)

B Cell Donors	Number of Microcultures	IgM^+ Cultures	RF^+/IgM^+ Cultures
Normal controls (n = 4)	544	149	0.7%
RA patients (n = 5)	2128	439	14.9%
pSS patients (n = 2)	1536	386	12.7%

Serial dilutions of the amplified product were dot blotted onto supported nitrocellulose membranes (BioRad, Hercules, CA, U.S.A.). Membranes were prehybridized in 55°C in 2 × SSPE, 5 × Denhardt's, 0.1% SDS, and denatured salmon DNA and hybridized with a biotinylated IgM-specific probe (AGGGTTGGGGCGGATG CACTC; Biotin-On, Phosphoramidite, Clonotech, Palo Alto, CA, U.S.A.) overnight. The membranes were then washed in 2 × SSC + 1% SDS at 45°C for 10 min, 55°C for 5 min, and 0.2 × SSC + 0.1% SDS at 65°C for 5 min. Membranes were blocked, incubated with streptavidin-alkaline phosphatase (DAKO, Carpenteria, CA, U.S.A.) and then developed using 5-bromo-4-chloro-3 indolyl phosphate and 4 nitro-blue tetrazolium chloride in a buffer containing 100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$, and pH 9.5. The blots were scanned using an AMBIS optical imaging system (San Diego, CA, U.S.A.). Amplified products were adjusted to contain equal amounts of IgM sequences, dot blotted, and hybridized with V3-21 (CCATTAGTAGTAGTAGTAGTTA) and V3-23–specific (CTATTAGTGGTAGTGGTGG TAG) biotinylated probes. The blots were hybridized, washed at 45 and 55°C, and developed as described above. The probes were specific for the appropriate VH sequences as determined on VH3 gene sequences derived from clonal B cell populations (data not shown).

Statistical Analysis

The distribution of VH, JH, and DH gene segment usage in the different B cell population was compared using a Pearson chi-square test. The frequencies of individual germline gene segment usage were compared by chi-square test or Fisher's exact test if appropriate.

RESULTS

Generation of RF-Producing B Cell Clones

To correlate antibody specificity, molecular characteristics of the IgM heavy chain and functional response to T cell signals, we have used a culture system in which clonal B cell populations were expanded in the presence of anti-CD3–activated T helper cells. To control for diversity of T helper cells, all experiments were done with a single T helper cell clone. B cells derived from patients and normal donors were examined in parallel cultures. Supernatants of established cultures were tested for antigen reactivity. As given in Table 1, secretion of IgM was detected in about 25% of the microcultures, which corresponded to a B cell cloning efficiency of 1:4 to 1:5. In healthy individuals, 0.7% of these IgM antibodies had Fc-binding specificity characterizing them as RF. The frequency of RF^+ secreting B cells was 20-fold increased in rheumatoid patients. Patients with pSS shared with RA patients the increased frequencies in RF^+ B cells.

Specificity of RF^+ B Cells Isolated from the Peripheral Blood of RF^+ Patients

To address the issue whether the RF produced by B cell clones from patients with pSS and RA represented monoreactive anti-Fc antibodies or polyreactive natural autoantibodies, supernatants from 42 B cell clones with RF activities from RA patients and 26 clones from patients with pSS were tested on a panel of soluble antigens. Only 8 of the 68 antibodies were truly polyreactive and had a broad cross-reactivity pattern (Table 2). Forty-five antibodies were monospecific for Fc fragments, and, within the panel of antigens tested, no cross-reactivity was observed. These data suggest that the majority of RF produced under these culture conditions did not

TABLE 2. Cross-reactivity pattern of rheumatoid factors

No. of Clones	Antibody Reactivity					
	IgG Fc	Actin	Keratin	ssDNA	Thyroglobulin	Tetanus Toxoid
2	+	+	+	+	+	+
1	+	+	+	+	+	−
1	+	+	+	−	−	+
1	+	+	−	−	+	+
1	+	−	+	+	+	−
1	+	−	+	+	−	+
1	+	−	+	+	−	−
1	+	+	−	−	−	−
4	+	−	+	−	−	−
3	+	−	−	+	−	−
3	+	−	−	−	+	−
4	+	−	−	−	−	+
45	+	−	−	−	−	−

represent natural autoantibodies. Patients with RA and pSS exhibited the same pattern of specificities. Twenty-four of the monospecific RF were derived from RA patients and 21 from patients with pSS.

Molecular Characteristics of Immunoglobulins with RF Reactivity

To identify the molecular characteristics of immunoglobulins with RF reactivity, VH gene family usage was analyzed in RF^+ and RF^- B cell clones derived from normal donors, RA patients, and pSS patients. The results are shown in Table 3. One hundred twenty-seven B cell clones from normal individuals were studied, all of them were negative for RF reactivity. Among these 127 B cell clones, the VH3 gene family was predominantly used. The distribution of VH gene families was not different from that in 113 RF^- B cell clones from RA patients, while it significantly differed from the VH distribution in RF^+ B cells ($p = 0.001$). Of the RF secreting B cells derived from RA patients, 77% belonged to the VH3 family, compared with 50% in the RF^- B cells from RA patients and 42% in healthy individuals. Similarly, VH3 elements were overrepresented in RF^+ B cells from pSS patients.

VH3 is the most complex family of gene segments with 37 different variants mapped so far. To address the question whether a correlation exists between germline diversity and RF speci-

TABLE 3. VH gene segment usage in IgM heavy chains from RF^+ and RF^- B cells

Donors	Specificity	No. of Clones	Frequency (%)						*p* Patients Versus Normal Controls
			VH1	VH2	VH3	VH4	VH5	VH6	
Normal controls	RF^-	127	30.7	3.9	42.5	8.7	12.6	1.6	N/A
RA patients	RF^-	113	23.0	3.5	50.4	16.8	4.4	1.8	NS
	RF^+	60	13.3	1.7	76.7	3.3	3.3	1.7	0.001
pSS patients	RF^-	82	24.4	2.4	43.9	12.2	11.0	6.1	NS
	RF^+	47	21.3	2.1	61.7	6.4	4.3	4.3	NS

TABLE 4. Usage of VH3 gene segment subtypes in IgM heavy chains from RF^+ and RF^- B cells

Donors	Specificity	No. of Clones	Frequency (%)					p Patients Versus Normal Controls
			V3-23	V3-30	V3-21	V3-15	Others	
Normal controls	RF^-	33	39.4	27.3	0.0	3.0	30.3	N/A
RA patients	RF^-	34	17.6	20.6	5.9	8.8	47.1	NS
	RF^+	31	16.1	13.0	19.4	16.1	35.5	0.007
pSS patients	RF^-	27	18.5	25.9	7.7	3.7	44.4	NS
	RF^+	27	11.1	37.0	14.8	0.0	37.0	0.03

ficity, we determined VH3 subtypes in $VH3^+$ B cell clones in the RF^- B cells from normal individuals, the RF^- and RF^+ B cells from RA patients, and in the RF^- and RF^+ B cells in pSS patients. The results are summarized in Table 4. Two germline gene segments, V3-23 and V3-30, were dominant in B cell clones established from normal individuals. These two germline segments accounted for 67% of the $VH3^+$ B cell clones analyzed. V3-23 and V3-30 were less prominent in the population of RF producing B cells from RA patients. Only 29% expressed a V3-23 or V3-30 gene segment. Two VH3 segments, V3-21 and V3-15, were more frequent in the repertoire of RF^+ B cells. In particular, V3-21, which was the most frequent VH3 element in RF^+ B cells from RA patients, was not detected in B cell clones from normal individuals. The distributions of VH3 gene segment usage in normal individuals and RF^+ B cells of RA patients were significantly different at the $p = 0.007$ level. The biased usage of VH3 subtypes correlated with the RF specificity of the antibodies, however, a similar but less pronounced trend was also evident for the RF^- B cells from RA patients. The V3-23 segment was generally underrepresented in RA B cell clones irrespective of their RF reactivity. In contrast to normal individuals, $V3\text{-}21^+$ B cell clones were found in the population of RF^- B cells from RA patients, albeit at a more less frequency than in RF^+ B cells.

In summary, B cells from RA patients express a unique repertoire of VH3 gene segments characterized by a reduction in V3-23 usage and the presence of V3-21. This fingerprint is present in RF^+ and in RF^- B cells of RA patients. However, there is a marked increase of V3-21 expression in RF^+ B cells, suggesting that an association exists between V3-21 and RF specificity.

This interpretation is further supported by the finding that RF^+ B cell clones derived from patients with pSS also used the V3-21 gene segment more frequently. The VH3 repertoire of RF^+ B cell clones from pSS patients differed significantly from the distribution in the RF^- population of normal controls ($p = 0.03$). Again, a similar trend was seen for the RF^- B cell clones derived from pSS patients, however, the shifts were much less pronounced (Table 4). Compared to RF^+ B cell clones from RA patients, pSS patients used the V3-30 gene element more frequently. Our data suggest that the VH repertoire of B cells from patients with RA and pSS which are responsive to the T cell derived stimuli are different compared with normal controls. These differences are shared between the two diseases and closely relate to the high number of RF secreting cells.

The five B cell populations (RF^- B cell clones of normal individuals, and RF^- and RF^+ B cell clones from patients with RA and pSS) were also compared for D and J gene segment usage (Tables 5 and 6). All sets of B cells preferentially utilized the JH4 gene segment for assembly of the Ig molecule. Between 50 and 70% of all B cell clones transcribed the JH4 gene segment. There was no significant differences in the distribution of JH gene segment usage.

D elements could be assigned to approximately 90% of the sequences. The D gene segment usage was heterogeneous and involved all different families with the exception of DHQ52. Again, there was no obvious difference in the distribution of D gene segment usage when the B cells from normal individuals and patients with and without RF specificity were compared.

Comparison of the amino acid sequences of the third complementarity determining region (CDR3) did not reveal any sequence homology in the B cell clones with RF activity (data not

TABLE 5. JH gene segment usage in VH3$^+$ IgM antibodies

Donors	Specificity	No. of Clones	Frequency (%)					
			JH1	JH2	JH3	JH4	JH5	JH6
Normal controls	RF$^-$	33	3.0	6.1	6.1	54.5	15.2	15.2
RA patients	RF$^-$	34	0.0	2.9	5.9	55.9	8.8	26.5
	RF$^+$	31	0.0	3.2	3.2	71.0	3.2	19.4
pSS patients	RF$^-$	24	4.2	0.0	16.7	50.0	12.5	16.7
	RF$^+$	23	0.0	13.0	17.4	52.2	4.3	13.0

shown). VH gene segment sequences were compared with the assigned germline gene segment sequences, and the mutations were analyzed to address the question of whether RF from either patients with RA or with pSS displayed evidence for somatic mutation. In all populations studied, the number of mutations were small. In about 90% of all sequences, either none or only one mutation was identified. The mutations found were randomly distributed, were not clustered in the CDR regions, and were not enriched for replacement mutations. No differences were seen between RF$^-$ and RF$^+$ B cell clones. Seventy-two percent of all RF$^+$ B cell clones from RA patients did not have any mutation in the V region and 14% had only a single mutation. These results were not different from the B cell clones without RF activity (60 and 27%, respectively). Results in the pSS cohort were very similar with no mutation in 59% of the RF$^+$ and 60% of the RF$^-$ clones and one mutation in 27% of the RF$^+$ and 25% of the RF$^-$ B cells.

V3-21 B Cells Are Not Deleted from the Repertoire of Normal Donors

The presence of V3-21$^+$–expressing B cells distinguished normal individuals from patients with RA and pSS. It is possible that patients and normal individuals differ in the germline repertoire. A second possibility is that V3-21$^+$ B cells are preferentially deleted because they correlate with RF specificity and therefore have a higher potential to be autoreactive. To address these possibilities, B cells were purified from PBMC of 10 normal individuals and 10 RA patients and were semiquantified for the expression of V3-23 and V3-21 sequences. RNA was amplified by RT-PCR with a VH3-IgM–specific primer set. The amplification product was adjusted for equal amounts of immunoglobulin sequences by hybridization with an IgM-specific probe. The adjusted material was then hybridized with oligonucleotides specific for V3-21 and V3-23. Specificity of both oligonucleotides was established in hybridization assays with VH3 sequences derived from

TABLE 6. DH gene segment usage in VH3$^+$ IgM antibodies

Donors	Specificity	No. of Clones	Frequency (%)									
			DXP	DA	DLR	DK	DN	DM	DHF16	DIR	DHQ52	Unknown
Normal controls	RF$^-$	33	39.4	0.0	21.2	6.1	18.2	12.1	0.0	9.1	0.0	6.1
RA patients	RF$^-$	34	38.2	2.9	11.8	17.6	20.6	0.0	5.9	2.9	0.0	8.8
	RF$^+$	31	35.5	6.5	6.5	22.6	16.1	3.2	9.7	0.0	0.0	6.5
pSS patients	RF$^-$	24	54.2	0.0	8.3	8.3	4.2	4.2	4.2	4.2	0.0	20.8
	RF$^+$	23	30.4	4.3	34.8	13.0	8.7	0.0	8.7	0.0	0.0	4.3

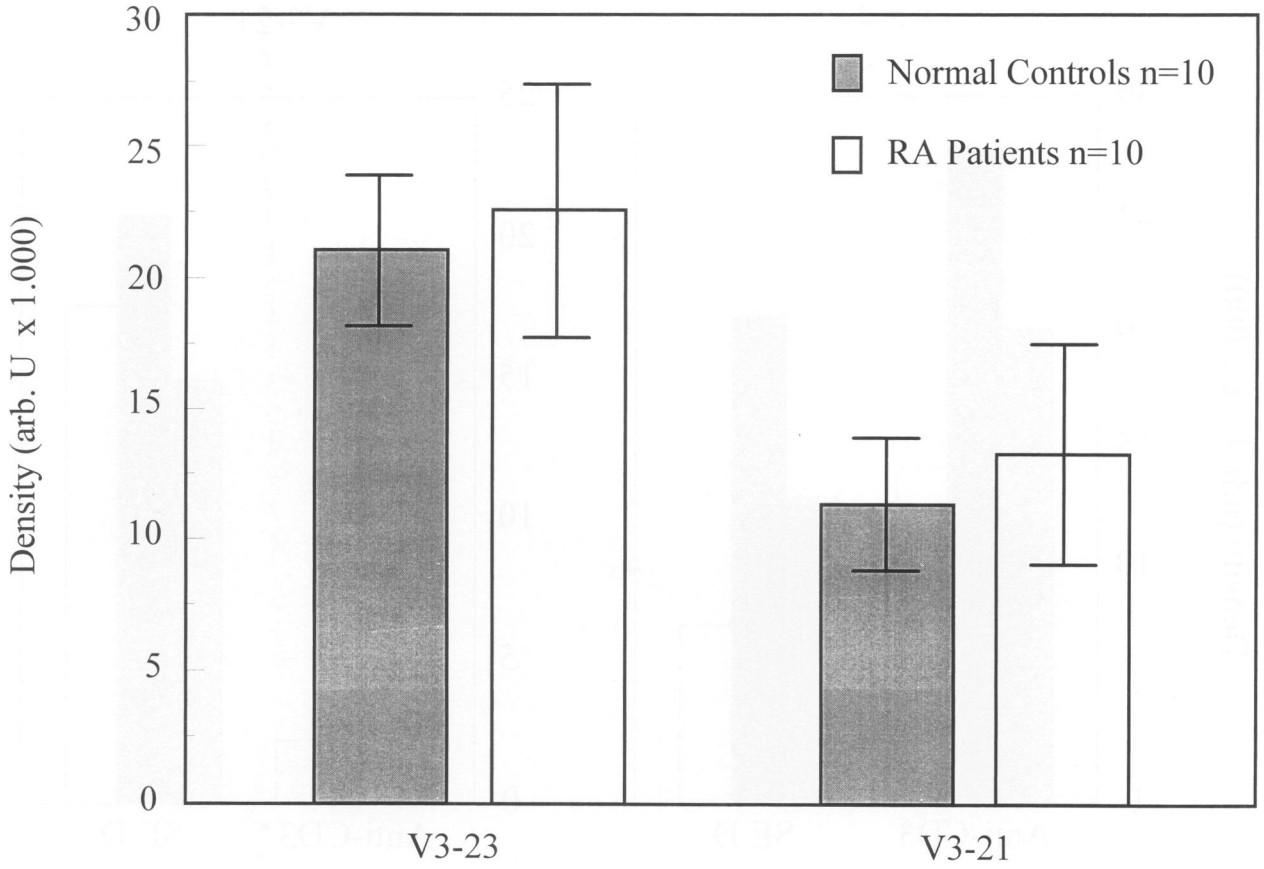

FIG. 1. Expression of V3-23 and V3-21 transcripts in unstimulated peripheral B cells of normals and RA patients

Peripheral blood lymphocytes were isolated from 10 normal donors and 10 RA patients. cDNA was amplified with VH3 and IgM specific primers. Amplified products were adjusted for equal amounts of IgM transcripts, dot blotted onto nitrocellulose membranes, and hybridized with biotinylated oligonucleotide probes specific for V3-21 or V3-23. The binding of the oligonucleotides was semiquantified using an Ambis system. Average densities of the V3-23 and V3-21 probes bound to VH3-specific transcripts from normal donors and RA patients are given.

sequenced B cell clones. The oligonucleotide hybridization assays were scanned using an AMBIS imaging system. Results are summarized in Fig. 1. All 20 donors (10 normal individuals and 10 RA patients) expressed the V3-21 as well as the V3-23 transcripts. While variations in the level of expression of V3-21 and V3-23 were detected in different individuals, there was no correlation with the presence of RA. The mean density for both VH3 elements was not different when normal donors and RA patients were compared (Fig. 1). These results demonstrate that V3-21$^+$ B cells exist in the repertoire of normal individuals but apparently are not activated when cocultured with anti-CD3–stimulated helper T cells.

Nonresponsiveness of V3-21 B Cells Derived from Normal Donors Can Be Overcome

Our data indicated that V3-21$^+$ B cells are present in the repertoire of normal individuals but they remained nonresponsive to anti-CD3–stimulated T cell clones. To further address this hypothesis, we explored different stimulation conditions with the goal to identify stimuli that could activate V3-21 B cells from normal individuals. We have recently described that the bacterial superantigen SE D can induce the preferential production of antibodies with RF specificity. Molecular analysis has indicated that the B cell population responsive to SE D includes V3-21$^+$ B

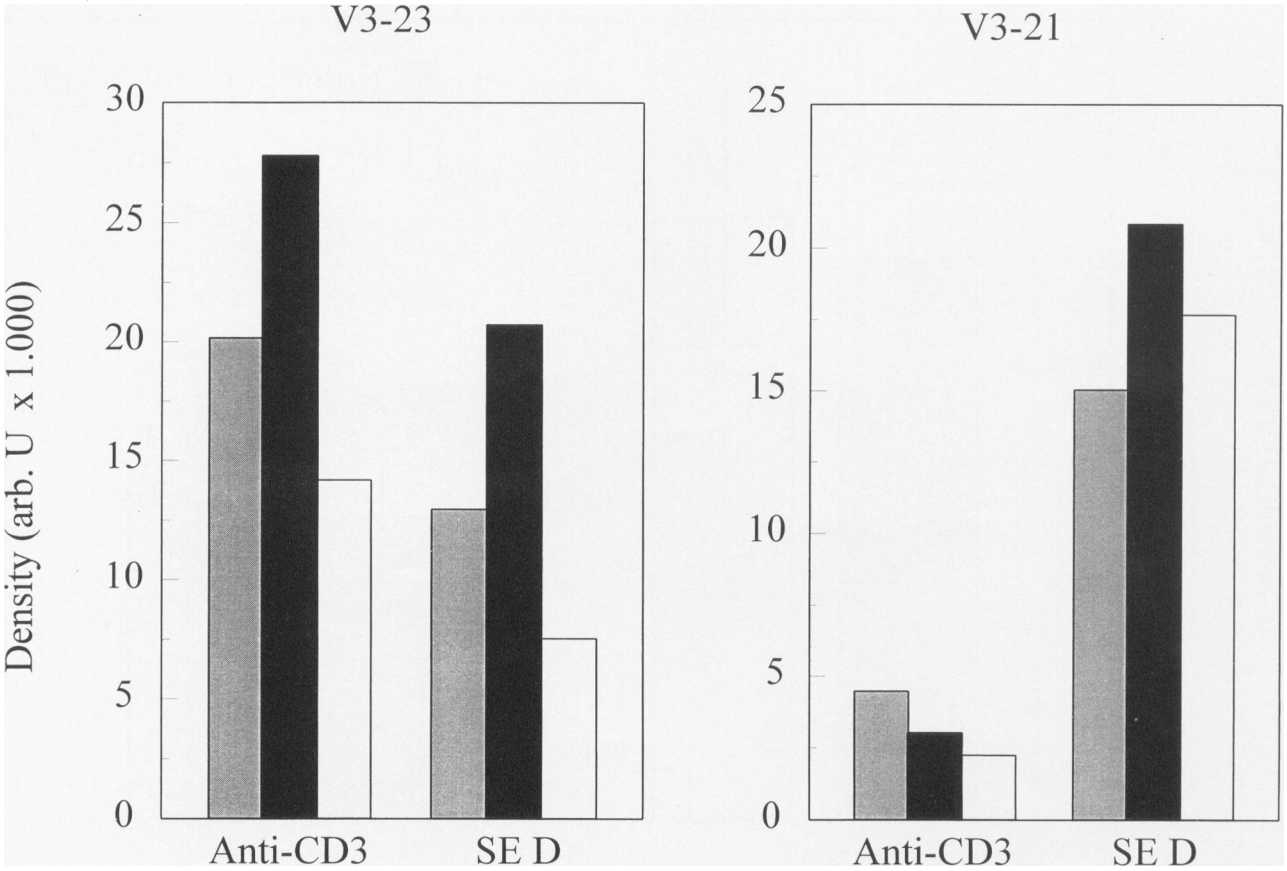

FIG. 2. Stimulation with the bacterial superantigen SE D activates V3-21$^+$ B cells which are nonresponsive to anti-CD3–driven T cells

B cells were isolated from the peripheral blood of three donors and cocultured with either anti-CD3–activated LAB1-10 T cells or LAB1-10 and SE D. Cells were harvested after 20 days. cDNA was amplified with VH3- and IgM-specific primers and the concentration of V3-23 and V3-21 transcripts were semiquantified as described in Fig. 1. V3-23$^+$ B cells were activated by both, anti-CD3–stimulated as well as superantigen-stimulated T cells. V3-21$^+$ B cells were nonresponsive to anti-CD3–driven T helper cells but responded in the presence of SE D. (■, Donor 1; ▨, Donor 2; □, Donor 3).

cells. We therefore compared the proliferation of V3-21$^+$ and V3-23$^+$ B cells under the two culture conditions, coculture with anti-CD3–activated T cells and coculture with T cells and SE D. Microcultures were harvested after 20 days and expression of V3-23 and V3-21 sequences were semiquantified by VH3-specific PCR and subsequent oligonucleotide-specific hybridization. Figure 2 shows the results from three normal individuals. Both, stimulation in the presence of anti-CD3–activated T cells and stimulation with SE D resulted in the induction of high concentrations of V3-23 transcripts. A different picture emerged for V3-21$^+$ B cells. While V3-21 B cells essentially disappeared from the cultures which were anti-CD3 driven, they proliferated extensively in the presence of SE D. Thus, the activation with SE D can overcome nonresponsiveness of V3-21$^+$ B cells in normal individuals. These B cells are present in the B cell repertoire of normal healthy individuals but remain unresponsive toward helper signals provided by anti-CD3–activated T cells.

DISCUSSION

Production of RF represents a major immunological abnormality in patients with RA (1–3). Although it has been speculated that RFs are antibodies directed against a disease-relevant antigen, the characteristics of the RF response lends only limited support to this model. RFs are highly heterogeneous in their antigen specificity,

are mostly of the IgM subtype, and usually do not increase in titer with chronic progression of the disease. Data presented here indicate that RA patients carry a subset of B cells which is altered in its responsiveness toward stimulatory signals. B cells with the potential to secrete RFs are present in normal individuals in frequencies which are indistinguishable from those in RA patients. However, in nonrheumatoid donors, these B cells remain unresponsive to signals provided by anti-CD3–stimulated T cells. In RA patients, these signals are sufficient to induce clonal expansion of these B cells and antibody secretion.

PBMC from RA patients have a high frequency of B cells secreting RF which can be expanded in cultures stimulated with activated T cell clones (30). Here, we report that the IgM secreted by these B cell clones has molecular characteristics that allowed the study of the function of this B cell subset in normals and in RA patients. RF^+ B cells display a nonrandom usage of VH gene segments with a marked overrepresentation of VH3. More importantly, the selective expression of the V3-21 gene segment in RA patients provides an opportunity to study RF^+ B cells. V3-21^+ B cell clones were exclusively isolated from patients. We have not been able to establish V3-21^+ B cell clones from a normal donor after B cell stimulation with anti-CD3–activated T cell clones. The exclusive expression of V3-21 in patients' B cell clones raised the question whether such B cells were present in the repertoire of normals. Experiments demonstrating IgM transcripts using the V3-21 gene segment in unstimulated B cells from normal individuals demonstrated that patients do not have a unique germline VH repertoire and that normals do not delete V3-21^+ B cells. Our data also suggest that the population of V3-21^+ B cells is not expanded in patients. Concentrations of V3-21–specific transcripts were similar in normal controls' and patients' B cells. Furthermore, V3-21^+ B cells derived from peripheral blood of normal controls can be stimulated to proliferate and to secrete antibodies with RF specificity by coculturing them with T cells and the bacterial superantigen SE D. The frequencies of RF-producing B cells after SE D stimulation are very similar in the peripheral blood of RA patients and healthy donors. Thus, the responsiveness, rather than the presence, of V3-21^+ B cells distinguishes healthy individuals and RA patients.

The association of V3-21 with RF specificity extends beyond RA. Patients with pSS, another disease characterized by RF production, share with RA patients a high frequency of RF-producing B cells which respond to anti-CD3–activated T cells. Again, RF-producing B cells frequently utilized a V3-21 gene segment. The finding that RF activity was a feature of V3-21 B cells, irrespective of CDR3 polymorphism and JH gene segment usage in both diseases, indicated that the protein structure encoded by the V3-21 gene segment is directly involved in conferring antigen specificity. Usage of the V3-21 gene segment has so far not been associated with RF specificity of immunoglobulins in RA patients. However, available sequence studies on RF^+ B cells have almost exclusively utilized EBV-transformed B cells, and EBV transformation may skew the repertoire by selectively targeting certain B cells. Apparently, the increased responsiveness of V3-21^+ B cells is shared by RA and pSS, suggesting that there may be a common pathogenetic mechanism underlying the RF production in these two distinct diseases.

Antibodies with RF specificity in normal individuals and also in RA patients frequently represent polyspecific antibodies or natural autoantibodies (31). Such natural autoantibodies are often considered to be secreted by $CD5^+$ B cells (32,33). The RFs studied here had characteristics which distinguished them from natural autoantibodies. Two thirds of the RFs were monospecific and did not cross-react on a panel of randomly selected antigens. Furthermore, expression of V3-21 mRNA was a feature of B cell clones derived from $CD5^+$ and $CD5^-$ B cells (data not shown). In summary, the experimental results support the model that a subpopulation of B cells circulates in the peripheral blood that is generally unresponsive to signals provided by polyclonally activated T helper cells. This B cell subpopulation is characterized by a nonrandom usage of VH gene segments and a bias for RF specificities. In disease states associated with RF production, these B cells change their reaction pattern and acquire responsiveness to antigen-nonspecific helper cell signals.

The mechanism underlying the distinct responsiveness of these B cells in RA is unclear and may include genetic regulation as well as exogenous stimuli. The coculture system that was employed in our studies utilized a cell-cell interaction between B cells and activated T cells to polyclonally stimulate B cells. It has been shown that plasma membranes of activated T helper cell clones can substitute for intact T helper cell clones in this system stressing the importance of

cell surface molecules (34). Although other costimulatory molecules may be involved in the B stimulation, the cross-linking of the CD40 molecule by CD40 ligand is the most important interaction for T cell induced B cell clonal expansion (35). Apparently, the concerted action of CD40 signaling, lymphokines, and costimulatory molecules is insufficient to trigger the entry of RF-producing B cells into the cell cycle in normal individuals, while these B cells are responsive in RA patients independent of exogenous antigen. Functional consequences of CD40 signaling in B cells are beginning to be understood and are probably dependent on the B cell differentiation stage and B cell type. In normal individuals, the V3-21$^+$ B cells are apparently anergized by the presence of ubiquitous IgG and the absence of a cognate T-B cell interaction as introduced by the recognition of a peptidic antigen on the surface of B cells serving as APC (36). However, these V3-21$^+$ B cells in normal individuals are responsive to exogenous stimulation as documented by the experiments utilizing the superantigen SE D. It is possible that SE D is directly recognized by V3-21 utilizing immunoglobulins and therefore not only acts as a T cell superantigen but also as a conventional B cell antigen in our culture system (17). We have previously described that SE D is unique among the staphylococcal enterotoxins in that it selectively induces RF production and that the VH repertoire of B cells responsive to SE D is skewed, reminiscent of the stimulation by other bacterial antigens (15,37–39). It is therefore possible that previous antigenic stimulation in RA patients has rendered these B cells responsive to polyclonal stimulation by a CD40-CD40 ligand interaction and subsequent secretion of RF.

There are at least two lines of evidence that would support the notion that the induction of RF in patients with RA is an antigen-specific event. First, several reports have shown that somatic mutation and affinity maturation occurs in immunoglobulins with RF specificity derived from patients with RA (40–45). Second, the production of RF appears to be an HLA-DR–associated phenomenon. HLA-DR alleles with a lysine substitution in position 71 of the β1 chain are specifically enriched in RF$^+$ patients (46). Conversely, RA patients lacking RF production mostly express HLA-DRB1 variants with an arginine substitution in position 71. In summary, RF synthesis appears to be an HLA-DR–linked phenomenon, probably introduced through the HLA restriction of selected helper T cells which can promote the growth and differentiation of RF$^+$ B cells. However, the V3-21$^+$ immunoglobulin genes we have sequenced from RA and pSS patients were all of the IgM isotype and did not show somatic mutation as would have been expected after antigen-specific selection. This finding is consistent with most reports on RF sequences indicating that somatically mutated RFs represent only a small fraction of the RF in RA patients (47). It remains to be clarified whether the loss of tolerance characteristic for V3-21$^+$ B cells in RA patients, but not in normals, is an acquired defect or a feature of the naive B cell repertoire. Studies in unaffected family members of patients with RF$^+$ disease could provide insight into whether genetic mechanisms contribute to the described changes in the functional B cell repertoire.

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