Clin Exp Immunol 1993; 92:404–411

The B cell repertoire of patients with rheumatoid arthritis. Frequencies and specificities of peripheral blood B cells reacting with human IgG, human collagens, a mycobacterial heat shock protein and other antigens

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(Accepted for publication 23 February 1993)

SUMMARY

Using a potent *in vitro* limiting dilution culture system, we have activated human peripheral blood B cells to proliferate and to differentiate into antibody-secreting cells (ASC). Under these conditions 25–100 % of B cells are clonally expanded and produce IgM, IgG or IgA. Culture supernatants were tested for antibodies binding to human IgG-F_C fragments (RF), the 65-kD heat shock protein of *Mycobacterium bovis* (hsp60), human collagens type I, II, IV, V, transferrin, lactoferrin, albumins, and gelatine. All blood samples contained precursors of ASC (p-ASC) able to produce IgM binding to these antigens in frequencies above 0·03% of B cells. Most interestingly, a significant difference exists between rheumatoid arthritis (RA) patients and controls, concerning the relative frequencies of p-ASC able to produce monospecific or multireactive RF. Whereas most p-ASC(RF) in RA patients are monospecific (mean ratio 3·7), most p-ASC(RF) in healthy control persons are cross-reactive with at least one of five other antigens tested (mean ratio 0·2). The data suggest a disease-specific expansion of p-ASC committed to the production of monospecific rheumatoid factors.

Keywords B cell repertoire rheumatoid factor heat shock protein autoantibodies collagen antibodies rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is believed to be an autoimmune disease mainly because of its association with certain HLA class II alleles [1], local infiltration of T and B lymphocytes [2] and the presence of autoantibodies. Serum antibodies to IgG (rheumatoid factors (RF)) are enhanced in patients with RA compared with healthy controls [3]. Presence of antibodies to collagen type II or the 65-kD heat shock protein hsp60 of *Mycobacteria* has been noted in several studies [4–7]. Collagen type II (CII) as well as the mycobacterial hsp60 do evoke arthritis in animal models [5,8,9]. Both autoantigens are present in the joints of RA patients [4,10]. In addition, plasma cells producing antibodies binding to human IgG-F_C fragments or to CII were observed in the synovial membrane of RA patients [11–13]. Nevertheless, the role of any of these autoantigens in inducing or maintaining RA as an autoimmune disease is not yet understood.

Usually, selection and differentiation of B cells require binding of the antigen as well as the presence of helper T cells. In order ultimately to understand whether autoantigens are involved in driving the local inflammatory response within the affected joints of RA patients, we and others started to analyse the functional receptor repertoire of B and T cells in patients and controls, in peripheral blood (PB) and in synovial fluid (SF) and membrane (SM) [14–18].

Only within the last few years have methods become available that allow estimation of the frequencies of antigen-specific antibody-secreting cell (ASC) precursors (p-ASC) in man. Either Epstein–Barr virus (EBV) or a mutant mouse T lymphoma clone in the presence of lymphokines and phorbol myristate acetate (PMA) are used to activate peripheral blood B cells under limiting dilution conditions to grow and to differentiate [16,19]. We used the system introduced by Wen *et al.* [19] to analyse the frequencies and specificities of human B cells with the capacity to produce antibodies reacting with human IgG-F_C fragments (RF), human collagens, hsp60 of *Mycobacterium bovis* and a variety of other antigens in patients with seropositive RA and healthy control persons.

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PATIENTS AND METHODS

Study subjects

In this study, 27 seropositive patients with RA were analysed. Most of them were female ($n=20$). The mean age was 57 ± 12

Table 1. Frequencies and clone sizes of peripheral blood (PB) B cells secreting immunoglobulins after activation *in vitro* (p-ASC)

	p-ASC (IgM)	p-ASC (IgG)	p-ASC (IgA)
RA patients			
frequency (mean)	1/1·4	1/3·6	1/4
range	1/1–1/4	1/1–1/10	1/1–1/12
number of experiments	20	13	10
ng immunoglobulin/clone ($x \pm$ s.d.)	32±26	9±9·5	5±4·7
range	7–75	2–25	2–14·5
number of experiments	7	5	7
Control persons			
frequency (mean)	1/1·6	1/3·3	1/3·6
range	1/1–1/3·5	1/1–1/7	1/1–1/7
number of experiments	6	6	6
ng immunoglobulin/clone ($x \pm$ s.d.)	21±8	4·9±2·9	4·9±2·9
range	5–30	2–9	1·7–9
number of experiments	5	5	5

Frequencies are calculated on the basis of $CD19^+$ B cells. The clone size (ng immunoglobulin/clone) was determined as (ng immunoglobulin/exp. group)/(B cells/group × frequency of p-ASC).

years. Twenty-two patients were HLA typed; of these, 20 were $DR4^+$ or $DR1^+$. At the time of blood sampling, some of the patients were treated with non-steroidal anti-inflammatory or disease-modifying drugs ($n=9$), the remaining patients received glucocorticoids ($n=12$) and/or antimetabolic drugs ($n=6$). Control persons were either healthy blood donors (two buffy coats kindly provided by the Central Unit for Transfusion Medicine, Medical Centre, Freiburg, Germany), laboratory personnel ($n=3$), or patients suffering from cardiac or cardiovascular disease ($n=3$). Their mean age was 49±13 years.

Preparation and characterization of B cells

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll–Hypaque gradient centrifugation (Biochrom Seromed, Berlin, Germany) and depleted for T lymphocytes by rosetting with sheep erythrocytes (kindly provided by Dr. H. Mossmann, Freiburg). The remaining E^- cell population was characterized by staining with antibodies to CD3 (OKT3, ATCC), CD14 (Leu-M3, Becton Dickinson) or CD19 (IOB4, Dianova, Hamburg, Germany) and FITC-labelled goat anti-mouse immunoglobulin (Dianova), followed by analysis on a FACScan (Becton Dickinson) at the Max-Planck-Institute f. Immunobiology, Freiburg. In addition, B cells were characterized by cytoplasmic staining with FITC-labelled antibodies reacting with human IgM, IgG or IgA (Dianova), read on a fluorescence microscope (Axioskop, Zeiss, Germany) with filter combination 487909. Usually the population contained 10–50 % $CD19^+$ B cells, of which *ca* 1% were positive for cytoplasmic IgG or IgA, respectively.

Limiting dilution cultures

Limiting dilution (LD) cultures were performed as described [19]. Briefly, 100 μl of LD medium (LDM) containing 10 % of conditioned medium (CM) and 3 ng/ml PMA were placed into round-bottomed microtitre wells (Nunc, Denmark). EL-4 B5 cells (5×10^4/culture) (kindly provided by R. H. Zubler, Geneva, Switzerland) irradiated with 50 Gy (^{137}Cs source, IBL) were added in 50 μl LDM consisting of RPMI 1640 (Biochrom Seromed, Berlin, Germany), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml, Gibco BRL, Gaithersburg, MD), 5×10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 10% fetal calf serum (FCS) (a selected batch, heat inactivated; Böhringer, Mannheim, Germany). E^- cells were added in two series of two-fold dilutions ranging from 32 to 0·5 and 1000 to 60 cells/culture, set up in 24 or 40 replicates/concentration. Control cultures did not receive E^- cells. Cultures were incubated at 37°C for 10 days, supernatants taken and stored at −70°C. CM was prepared in bulk cultures of nylon wool-purified T lymphocytes of several healthy donors (kindly provided by the Central Unit for Transfusion Medicine, Medical Centre, Freiburg) stimulated at 1×10^6 cells/well in LDM supplemented with PMA (10 ng/ml) and phytohaemagglutinin (PHA) (5 μg/ml, Wellcome Diagnostics, Dartford, UK) for 36 h. Supernatants were centrifuged, filtered (0·22 μm) and stored in aliquots at −20°C.

Antigens

Antigens used as coats in ELISAs included human lactoferrin (Serva, Heidelberg, Germany), human transferrin (Böhringer), gelatine, bovine serum albumin (BSA; Behring, Marburg, Germany), human serum albumin (HSA; Behring), ovalbumin (OA; Sigma, Deisenhofen, Germany) and human IgG F_C fragments (Dianova). Human collagens type I, II, IV and V were kindly provided by K. von der Mark (Erlangen, Germany). They were prepared as described [20], and dissolved in 0·1 M acetic acid. Mycobacterial hsp60 was kindly donated by S. H. E. Kaufmann and B. Schoel (Ulm, Germany). *Escherichia coli* clone M1103 containing the gene of mycobacterial hsp60 was kindly provided by J. D. A. van Embden (Bilthoven, The

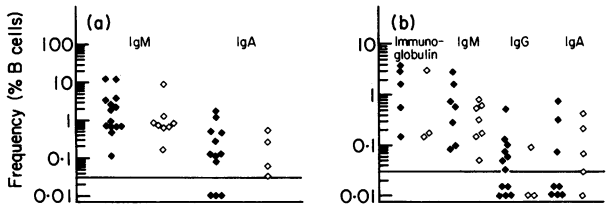

Fig. 1: Frequencies of peripheral blood (PB) precursors of antibody-secreting cells (p-ASC) with the capacity to produce rheumatoid factors (RF) (a) and antibodies binding to mycobacterial hsp60 (b). Culture supernatants were assayed for immunoglobulin binding to human IgG-F_c fragments (IgM, IgA) or hsp60 (IgM, IgG, IgA). The horizontal line indicates a frequency of 0·03 %, which is the lower limit of detection in these experiments. ◆ and ◇ represent frequencies estimated in rheumatoid arthritis (RA) patients and normal controls, respectively. Compare Table 2.

Netherlands). The soluble constituents of bacteria were purified as described [21].

Assays for antibody quantification and specificity

Antibodies present in tissue culture supernatants were assayed with ELISAs using goat anti-human IgM, IgA or IgG labelled with alkaline phosphatase as second antibodies (Dianova), and PNPP (Sigma) as substrate. ELISA plates (Nunc) were coated with antigens at 5–10 μg/ml for 1 h at 37°C or room temperature (collagens). Gelatine was then added to cover uncoated plastic sites. Further procedures followed standard protocols as described [22,23]. ELISA absorbance units (EAU) were read at a λ_T/λ_R ratio of 405 nm/490 nm (EAR 400 AT, SLT, Austria). For segregation analyses, individual culture supernatants were split into up to six aliquots and tested either on different antigens (to analyse specificity) or with different anti-immunoglobulins (to analyse immunoglobulin class switch) [23,24]. The amount of immunoglobulin/culture was determined in ELISAs using goat anti-human immunoglobulin as coat and dilutions of human IgM, IgG or IgA as standards (all reagents: Dianova). The amount of immunoglobulin secreted per B cell clone was calculated at low B cell concentrations using the formula: $\text{immunoglobulin}_{\text{clone}} = (\text{ng immunoglobulin/experimental group})/ (\text{B cells/group} \times \text{frequency of p-ASC})$.

Statistical analysis

EAU values were further processed using the program Statgraphics (Statistical Graphics Corp., Rockville, MD) on a Siemens PCD2 computer. Control groups without E^- cells were used to calculate the threshold $t1 = x + 3$ s.d. which served to differentiate between positive and negative cultures. Frequencies were estimated in experiments with ≥ 3 F_0 values $\neq 1{\cdot}00$ using χ^2 minimization as described [22,23,25]; only estimations with $P > 0{\cdot}05$ were accepted and are shown here. In this LD system, the limit of detection was set to a frequency of 1/3333 (= 0·03%). Segregation analyses were performed with cultures containing ≤ 1 B cell with the attribute of interest (i.e. $F_0 \geq 0{\cdot}37$). Instead of $t1$ here a second threshold $t2$ ($t2 = 2xt1$) was used to distinguish cultures with low EAU values from those with high EAU values. All frequencies are calculated on the basis of B cell input ($CD19^+$, cIg^+). Further statistical analyses were performed using the program Statgraphics.

RESULTS

Frequencies, switch and clone sizes of in vitro *activated PB B cells*

We have activated PB B cells of patients with seropositive RA and of healthy control persons in limiting dilution cultures. Day 10 supernatants of these cultures were assayed for the presence of IgM, IgG and IgA. The distribution of positive and negative cultures was analysed by Poisson statistics [24]. Table 1 summarizes the frequencies observed for p-ASC secreting IgM, IgG or IgA, respectively, after activation in culture. In several experiments the amount of antibodies was quantified and the amount produced per culture and per clone calculated. In general, most if not all PB B cells could be activated to grow, to differentiate and to produce antibodies. Frequencies of p-ASC estimated in experiments with PB B cells obtained from patients ($n = 20$) or controls ($n = 6$) were comparable, as were the resulting clone sizes. Both sets of data were similar to those published before [19].

The frequencies of p-ASC secreting IgM, IgG or IgA add up to more than 100 % of all $CD19^+$ B cells (Table 1), and the frequencies of p-ASC (IgG) and p-ASC (IgA) are higher than expected from the numbers of IgG^+ and IgA^+ PB B cells. These data suggested that most, but not all p-ASC (IgG or IgA) are derived from p-ASC (IgM) switching *in vitro*, as shown before [19]. This was confirmed in every individual experiment by segregation analyses (data not shown, [23, 24]).

Frequencies of PB B cells with the capacity to produce antibodies binding to RA-related antigens

To estimate the frequencies of PB p-ASC capable of producing antibodies binding to antigens which may be important in RA, we assayed culture supernatants for immunoglobulin (or IgM) binding to human IgG-F_C fragments, or mycobacterial hsp60. As p-ASC (IgM) frequencies were close to 1/1 B cell, p-ASC frequencies are expressed as percentage of B cells. Frequency data are individually shown in Fig. 1 and summarized in Table 2. Due to the limitations of the culture system, only relatively high frequencies could be estimated ($\geq 1/3333$ or $\geq 0{\cdot}03\%$ of B cells). Donors with no antigen-binding p-ASC above this threshold are therefore listed as 'negatives' in Table 2 (compare n_p/n_t). 'Negative' frequencies were arbitrarily set to a value of 0·03 % in calculations (Tables 2–6).

All donors contained relatively high frequencies of p-ASC (IgM)-producing antibodies binding to human IgG-F_C fragments or mycobacterial hsp60 ($> 1/500$ B cells). In many experiments also IgG or IgA antibodies binding to these antigens were determined (Table 2 and Fig. 1). p-ASC (RF/IgA) frequencies above 0·03 % were only detected in 75% of the RA patients, but all controls. p-ASC (hsp60/IgA) and p-ASC (hsp60/IgG) frequencies above 0·03 % were observed in 37·5% and 58·3% of the patients and 80% and 33·3% of the controls, respectively. In all groups large variations between individual frequencies were observed (Fig. 1). Although p-ASC frequencies specific for human IgG-F_c fragments or mycobacterial hsp60 were higher in the group of RA patients than in the control group, this difference was not statistically significant (Tables 2 and 6). As expected, frequencies of IgM-producing p-ASC of both specificities were significantly higher than the frequencies of IgG- or IgA-producing p-ASC of the same specificity (Tables 2 and 6).

Table 2. Frequencies of antigen-specific precursors of antibody-secreting cells (p-ASC) in seropositive rheumatoid arthritis (RA) patients and healthy control persons

Assay system		RA patients			Healthy controls		
			Frequencies (% of B cells)			Frequencies (% of B cells)	
Antigen	Antibody class	Sample size (n_p/n_t)*	Mean†	St. dev.	Sample size (n_p/n_t)	Mean	St. dev.
F_c fragment	IgM	14/14	1·48 *t*	4·15	8/8	0·91 *t*	2·97
	IgA	9/12	0·29 *p*	0·55	4/4	0·13 *t*	0·24
			0·17 *t*	0·52			
hsp60	Ig	5/5	1·06 *t*	1·51	3/3	0·43 *t*	1·71
	IgM	7/7	0·47 *t*	1·02	7/7	0·28 *t*	0·29
	IgA	3/8	0·27 *p*	0·35	4/5	0·12 *p*	0·19
			0·07 *t*	0·26		0·09 *t*	0·18
	IgG	7/12	0·09 *p*	0·18	1/3	0·09 *p*	—
			0·06 *t*	0·14		0·04 *t*	0·04

* Sample size is given as number of experiments with frequency estimates >0·03% (n_p) and number of experiments performed (n_t).

† Geometric means ± s.d. are calculated from all n_p (*p*) and n_t (*t*). In the latter case frequencies below 0·03% were set to 0·03%.

Table 3. Frequencies of precursors of antibody-secreting cells (p-ASC) recognizing various antigens not related to rheumatoid arthritis

	Per cent of B cells producing antibodies		
Antigen	IgM	IgG	IgA
BSA	2·00	0·17	0·12
HSA	0·61	<0·03	<0·03
OA	0·54	<0·03	<0·03
Gelatine	<0·03*	<0·03	<0·03
Lactoferrin	0·29	<0·03	<0·03
Transferrin	0·65	<0·03	<0·03
Fc-fragment	ND	ND	(2) 1·6†
hsp60	1·16	0·48	1·05
		(2) 0·53	(2) 0·76

* Only two cultures were positive. Therefore no frequency could be calculated.

† Data obtained in a second experiment are marked by (2).

BSA, Bovine serum albumin; HSA, human serum albumin; OA, ovalbumin; ND, not done.

Frequencies of PB p-ASC able to produce antibodies to antigens not suspected of being involved in RA

On several occasions, culture supernatants were tested on a variety of antigens not thought to be involved in RA, e.g. gelatine, serum albumins of several species, human lactoferrin and transferrin. Table 3 shows representative results obtained in two consecutive experiments with PB B cells of one RA patient. Here, as in other cases, we estimated relatively high frequencies of antigen-binding p-ASC (IgM) recognizing most antigens (>0·03 %), with the exception of gelatine-specific p-ASC (IgM), where only occasionally positive cultures were found. However,

Table 4. Segregation analysis of precursors of antibody-secreting cells (p-ASC) (RF/IgM) and p-ASC (immunoglobulin) binding to five other antigens at the clonal level

Specificity						Number of clones with the pattern indicated	
RF	hsp60	CI	CII	CIV	CV	RA2	NC2
Monospecific							
+	−	−	−	−	−	28	3
−	+	−	−	−	−	0	3
−	−	+	−	−	−	0	4
−	−	−	+	−	−	0	1
−	−	−	−	+	−	0	1
−	−	−	−	−	+	1	43
Crossreactive							
+	+	−	−	−	−	1	1
+	+	−	−	+	−	0	3
+	+	+	−	+	−	0	4
+	+	+	−	−	−	0	0
+	+	−	−	−	+	0	1
+	+	−	−	+	+	1	3
+	+	+	−	+	+	0	5
+	+	+	+	+	+	0	0
+	−	−	−	−	+	1	3
+	−	−	−	+	+	0	2
RF^-, several patterns						0	16
−	−	−	−	−	−	88	27
Total number of cultures tested						120	120

Supernatants of individual cultures were assayed for binding to the antigens indicated. For segregation analysis only groups with $F_0 \leq 0{\cdot}37$ were used, and classified as positive (+) or negative (−) according to *t*2 for all specificities. Compare Table 5.

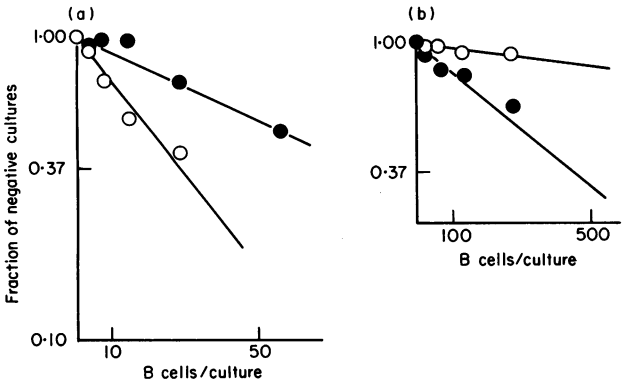

Fig. 2: Frequencies of monoreactive and multireactive precursors of antibody-secreting cells (p-ASC) (RF/IgM) as estimated in rheumatoid arthritis (RA) patient RA2 (a) and control person NC2 (b). ○, Monospecific, ●, multireactive p-ASC (RF/IgM), determined by analysing all culture supernatants derived from clones for binding to six antigens (compare Tables 4 and 5).

Table 5. Frequencies of precursors of antibody-secreting cells (p-ASC) (IgM) committed to produce monospecific or multireactive rheumatoid factors

	Frequencies of p-ASC (RF/IgM) (% of B cells)		
Person	Monospecific	Multireactive	Ratio
RA1	0·43 (0·16–0·70)	0·19 (0·13–0·20)	2·26
RA2	3·35 (2·22–4·48)	1·25 (0·85–1·67)	2·68
RA3	0·69 (0·55–0·83)	0·21 (0·12–0·29)	3·29
RA4	1·98 (1·02–2·91)	0·24 (0·17–0·36)	8·25
RA5	0·13 (0·05–0·22)	0·03 (0·01–0·07)	4·33
Mean*	0·76(1·34)	0·21 (0·49)	3·72 (2·41)
NC1	0·05 (0·01–0·09)	0·10 (0·05–0·16)	0·50
NC2	0·04 (0·01–0·07)	0·22 (0·15–0·29)	0·18
NC3	0·71 (0·09–1·34)	4·36 (2·84–5·89)	0·16
NC4	0·27 (0·04–0·49)	0·77 (0·36–1·18)	0·35
NC5	<0·03 (ND)	0·46 (0·26–0·66)	<0·07
Mean	0·10 (0·29)	0·51 (1·80)	0·20 (0·17)

* Geometric means (s.d.).

Frequencies of monospecific and crossreactive p-ASC (RF/IgM) are given for 10 individual seropositive rheumatoid arthritis (RA) patients or controls. The 95% confidence limits are given in brackets. In addition, the ratio between the frequencies of both types of p-ASC (RF/IgM) is calculated.

neither in this patient nor in other patients or control persons were p-ASC (IgG) or p-ASC (IgA) specific for HSA, OA, gelatine, lactoferrin or transferrin detected with frequencies above 0·03%.

Specificity of PB B cells producing antibodies to human IgG (rheumatoid factors)

The limiting dilution system used not only allows the estimation of frequencies, but also the analysis of antibody specificity. Here we analyse the specificity of p-ASC (RF/IgM). For this purpose, 10 experiments were set up with B cells of five RA patients and five control persons. Individual supernatants were tested for antibodies binding to any one of four to six different antigens (Table 4). All cultures positive for p-ASC (RF/IgM) from groups with $F_0 < 0{\cdot}37$ were regarded as clonal [24, 25]. In RA patients most of these clones were monospecific, whereas in normal controls most p-ASC (RF/IgM) were cross-reactive with at least one of the other antigens. As an example, we discuss data of patient RA2 and control NC2. In RA2, 31 cultures contained IgM binding to F_c fragments, of which only three cross-reacted with other antigens (one with hsp60, one with CV and one with hsp60, CIV and V). Thus, in this patient, 28 of 31 positive cultures were monospecific, i. e. 90·3 %. In NC2, 22 of 25 cultures containing IgM binding to F_c fragments cross-reacted with other antigens; thus only three of 25, i. e. 12 % of the positive cultures, were monospecific.

The data shown in Table 4 can also be used to determine frequencies of monospecific and multireactive p-ASC (RF/IgM). As an example, Poisson plots of the experiment with RA2 and NC2 are shown in Fig. 2. Table 5 gives a summary of all 10 experiments. In RA patients the frequency of monospecific p-ASC (IgM/RF) is three- to four-fold higher than the frequency of multireactive p-ASC (IgM/RF), whereas the contrary is observed in all controls: here the frequency of monospecific p-ASC (RF/IgM) is five-fold lower than the frequency of multireactive p-ASC (RF/IgM). In general, RA patients have higher frequencies of monospecific precursors and lower frequencies of multireactive precursors than controls. This difference is not significant (Table 6). In contrast, the ratios between the frequencies of monospecific and cross-reactive p-ASC (RF/IgM) differ significantly from each other.

DISCUSSION

Autoantigens involved in the autoimmune response may be expected to increase the frequency of specific precursor cells due to an antigen-driven selection process [26]. Indeed, B cells committed to the production of IgG antibodies specific for insulin, thyroglobulin or DNA were observed in higher frequencies in patients with insulin-dependent diabetes mellitus, Hashimoto's thyroiditis or systemic lupus erythematosus than in healthy controls [16,27]. In RA patients there is some evidence for higher frequencies of B cells committed to the production of IgM rheumatoid factors [17,18,28,29]. The absolute numbers of p-ASC (RF/IgM) reported by several authors for individuals vary between 0·03% and 3% (healthy controls) and 0·005% and 24% (RA patients) of B cells.

We used the EL-4 system introduced by Wen *et al.* [19] and tested the supernatants of activated B cells for reactivity to human IgG-F_C fragments, mycobacterial hsp60 and additional antigens. In our hands this technique works as efficiently as described, concerning the frequency of activation (close to 100 %), the amount of immunoglobulin produced and the induction of switch *in vitro* (Table 1). B cells from seropositive RA patients can be stimulated and respond in the same way as those of healthy controls.

Individual frequencies of p-ASC (RF) and p-ASC (hsp60) showed a large degree of variation, reaching a maximum of 12·5 % of B cells committed to produce IgM RF in two patients (Fig. 1). Values estimated in RA patients and healthy controls follow

Table 6. Comparisons of precursors of antibody-secreting cell (p-ASC) frequencies

p-ASC	Parameter	d.f.*	F critical value	F ratio	Significance level
IgG-F_C fragment	Immunoglobulin class†	1,24	4·26	5·031	0·0344
hsp60	Immunoglobulin class	3,28	2·95	7·023	0·0011
IgG-F_c fragment, IgM	Health‡	1,20	4·35	0·608	0·4531
IgG-F_c fragment, IgA	Health	1,14	4·60	0·324	0·5840
hsp60, IgM	Health	1,12	4·75	1·640	0·2245
hsp60, IgG	Health	1,13	4·67	0·270	0·6178
hsp60, IgA	Health	1,11	4·84	0·001	0·9739
Monospecific RF, IgM	Health	1,8	5·32	3·203	0·1113
Multireactive RF, IgM	Health	1,8	5·32	0·920	0·3756
Ratio mono/multi RF, IgM	Health	1,8	5·32	13·026	0·0069

* d.f., Degrees of freedom.

† IgM and IgA or immunoglobulin, IgM, IgG and IgA.

‡ Rheumatoid arthritis (RA) patients and healthy controls.

Frequencies shown in Tables 2–5 are compared by analysis of variance according to the parameters immunoglobulin class or health. Frequencies below 0·03% of B cells were arbitrarily set to 0·03%. Calculated F ratios are underlined, if they indicate a significant deviation from a normal distribution at $\alpha = 0{\cdot}05$.

a normal distribution (data not shown), and fall into the range of frequencies reported by others [17,18,28,29]. While we observed p-ASC (IgM) reactive to human IgG-F_C fragments or hsp60 in all samples, specific p-ASC (IgG) and p-ASC (IgA) could not be detected in all cases. Interestingly, p-ASC (RF/IgA) occurred in relatively high frequency in all controls, but only 75% of RA patients; p-ASC (hsp60/IgA) in 80% of controls, but only 38% of patients, whereas p-ASC (hsp60/IgG) occurred in 1/3 controls (= 33%) and 7/12 patients (= 58%). All other antigens tested, with the notable exception of gelatine, were recognized by a relatively high number of B cells producing IgM after activation in culture (> 0·03%), but no p-ASC (IgG) or p-ASC (IgA) reacting with the autoantigens HSA, lactoferrin or transferrin were ever observed in frequencies above 0·03% of PB B cells (Table 3 and data not shown). To our knowledge, frequencies of p-ASC specific for mycobacterial hsp60 have not been estimated before. The high precursor frequency of IgM- as well as of IgG- and IgA- producing B cells emphasizes again the immunogenicity of stress proteins with highly conserved structure [8,9,30].

The most interesting aspect of the present study is the observation of a significant difference between RA patients and controls, concerning the specificity of B cells committed to RF production (Tables 5 and 6, Fig. 2). Clearly, a monospecific B cell population can be distinguished from a cross-reactive one. In all five RA patients monospecific p-ASC (RF/IgM) were more frequent than cross-reactive p-ASC (RF/IgM) (mean ratio 3·7), whereas in all five control subjects the contrary was the case (mean ratio 0·2). These results confirm and extend data of several authors indicating a prevalence of monospecific RF or of special RF idiotypes in RA patients [11,17,31–34]. Cross-reactivity most frequently occurred with hsp60 or/and collagen type V. Probably, such antibodies belong to the type of 'natural' multireactive antibodies described and discussed earlier [35,36]. They exist in all donors in relatively high frequencies.

We earlier described a similar phenomenon in the mouse system [23]. Erythrocyte-specific B cell precursor frequencies among lipopolysaccharide-reactive cells from three pairs of IgH-congenic high and low responder strains showed four- to five-fold differences, closely resembling the differences observed in bulk culture responses. In addition, we found that the V_H part of the IgH locus is responsible for the observed frequency differences, suggesting that V_H germ line genes directly influence the composition of the mature B cell repertoire. Monospecific rheumatoid factors in RA patients were shown to use germ-line genes [37,38]. Recently, polymorphisms in the human VIgH locus were described, including differences in germ-line genes known to be used in monospecific rheumatoid factors [38–40]. It was suggested that B cells expressing certain V-gene families may be expanded by superantigens [41,42]. Our data suggest that seropositive RA patients carry an IgH haplotype containing germ-line V_H genes used by a B cell population committed to the production of monospecific rheumatoid factors and expanded by a still unknown mechanism. Limiting dilution analysis may help to define this B cell population, its origin, specificity and significance for the development of rheumatoid arthritis.

ACKNOWLEDGMENTS

We are grateful to Drs S. H. E. Kaufmann and B. Schoel, Ulm and Dr K. von der Mark, Erlangen, for their generous gifts of mycobacterial hsp60 and human collagens, respectively, and other contributions. We thank Drs M. Tongio and A. Urlacher, Strasbourg, and A. Becking, Freiburg, for performing the HLA typing; Dr. H. Mossmann, Freiburg, Dr. R.H. Zubler, Geneva, and Dr J. v. Embden, Bilthoven, for the gift of reagents. We are grateful to Dr H. Beeser and his staff at the Central Unit for Transfusion Medicine, Freiburg, for providing us with material from blood donors. Dr. K. Eichmann gave us the opportunity to use the cell sorter at the Max-Planck-Institute for Immunobiology, Freiburg. Moreover, we feel obliged to him for his continuous support of our work

and many critical discussions. This work was supported by DFG grant Pe 151/10.

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