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Analysis of immunoglobulins secreted by hybridomas derived from rheumatoid synovia

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SUMMARY

Twenty-six IgG-secreting and eight IgM-secreting hybridomas were derived from the synovia of two patients with rheumatoid arthritis (RA). Hybridomas were obtained by fusing a heteromyeloma cell line, SPAZ-4 with synovial mononuclear cells that were not deliberately stimulated *in vitro*. Over 96% of the IgG-secreting hybridomas produced antibodies which belonged to the IgG1 subclass and showed lambda light chain predominance; the latter was not seen in IgM antibodies, where kappa light chains dominated by 3:1. All IgG antibodies were cationic. Synovial B cells were not exposed to extrinsic stimuli prior to fusion, therefore these results reflect the state of B cell activation and differentiation *in vivo*. Our results indicate that IgG-secreting B cells in the RA joint are under a selective influence which is, as yet, unidentified. One out of eight IgM-secreting and two out of 26 IgG-secreting hybridomas produced rheumatoid factors (RF). The IgM-RF specificity for IgG heavy chain subclasses was determined and showed that the monoclonal bound to IgG1, IgG2 and IgG4 but not IgG3 with exception of IgG3 Goe of the G3m (st) allotype, a profile typical of specificity for the Ga epitope. This monoclonal also distinguished a determinant in the Fc region of human IgG which was not present in rabbit IgG. The overall frequency of RF-secreting hybridomas we observed indicates that B cells committed to RF production in the synovium of a seropositive and a seronegative RA patient is below 10%.

Keywords human hybridomas synovial tissue rheumatoid arthritis immunoglobulin isotypes

INTRODUCTION

Chronic inflammatory reactions occurring in rheumatoid joints have an important immune component and are characterized by a massive cellular infiltration into the synovial membrane. Infiltrating lymphocytes are predominantly T cells, and plasma cells are also abundant despite a paucity of B cells. Locally synthesized and secreted immunoglobulins include rheumatoid factors (RF) and IgG antibodies (Smiley, Sachs & Ziff, 1968; Sliwinski & Zvaifler, 1970) resulting in the formation of immune complexes within the joint fluid, which are thought to contribute to the pathogenesis of rheumatoid arthritis (RA) (Zvaifler, 1973).

In order to understand the role of the B lymphocyte in the pathogenesis of RA, the specificity and biological activities of locally secreted synovial antibodies need to be determined. Previous studies have been directed at the analysis of synovial tissue culture supernatants and histological examination of synovial tissue sections. These studies suggest a restricted heterogeneity of antibodies secreted in the rheumatoid synovium, as evidenced by lambda light chain predominance (Epstein & Tan, 1966; Lindström, 1970; Munthe & Natvig, 1972), over-representation of particular IgG subclasses (Munthe & Natvig 1972; Hoffman, Goldberg & Smiley, 1982; Mellbye, Vartdal & Dobloug, 1984) and restriction in the isolelectric point of synovial IgG (Hoffman, Douglass & Smiley, 1984).

To investigate synovial B cells we have derived a panel of antibody-secreting hybridomas by fusing synovial mononuclear cells with a heteromyeloma fusion partner (SPAZ-4) and selecting hybridomas secreting IgG or IgM. Only dividing cells give rise to stable hybridomas, and since the mononuclear cells were not deliberately activated *in vitro* we have used this technique to immortalized cells activated *in vivo*. This has provided an opportunity to study B lymphocytes actively involved in the immune reaction occurring at the disease site. The heterogeneity of synovial antibodies has been examined by assessing the distribution of light chain type and IgG subclass and analysing the isoelectric focusing patterns (spectrotypes) of IgG secreted by the hybridomas. To determine RF secretion, all supernatants from IgG- and IgM-secreting hybridomas have been screened for reactivity with human IgG.

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MATERIALS AND METHODS

Rheumatoid synovial membranes

Synovial membranes were obtained during surgery from two patients with RA who satisfied the ARA criteria (Ropes *et al.*, 1959). Patient A was a 59-year-old woman with classical erosive seropositive RA, who had a total hip replacement. The duration of her disease was 6 years. At the time of surgery she was taking piroxicam, aspirin and salazopyrin. Patient B was a 38-year-old woman with polyarticular erosive seronegative RA who had a synovectomy of the knee. The duration of her disease was 13 years. At the time of surgery she was taking prednisolone, indomethacin and copraxamol.

Lymphocyte extraction

Minced synovial tissue was digested with a mixture of DNAse I (5 μg/ml (Sigma, Poole, UK) and collagenase type IV (20 μg/ml) (Sigma) for 2–3 h at 37°C with shaking. The digest was passed through nylon mesh and the filtrate centrifuged over Ficoll–Paque (Pharmacia, Bedfordshire, UK) for 30 min at 1500 rev/min. Mononuclear cells were removed from the interface, washed in HBSS (GIBCO, Paisley, UK) and cryopreserved in liquid nitrogen using an automated cell freezer (Planar Products, Middlesex, UK).

B cell fusion

All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium supplemented with 2 mM glutamine, 2 mM Na pyruvate and fetal calf serum (FCS) (all from GIBCO) at concentrations stated during the following procedures.

A heteromyeloma cell line (SPAZ-4) used as a fusion partner for human B cell hybridoma production was obtained from Dr Phil Lake, Sandoz Research Institute, NJ. SPAZ-4 is a non-immunoglobulin secreting, 8-azaguanine resistant cell line; its derivation has previously been described (Ostberg & Pursch, 1983). SPAZ-4 cells were grown to log phase growth in medium supplemented with 5% FCS, collected by centrifugation and washed twice in HBSS. Thawed synovial membrane mononuclear cells (40×10^6 and 19×10^6 from patients A and B, respectively) were washed twice in HBSS and mixed with SPAZ-4 cells in a 50-ml tube (Falcon, Becton Dickinson, Oxford, UK) in the ratio 5–10:1, respectively. Cells were fused by adding 1 ml 50% polyethylene glycol 1500 (PEG) (Boehringer Corporation, East Sussex, UK) at 37°C for 1 min while gently shaking the tube. PEG was diluted out by adding 10 ml HBSS at 37°C gradually to the cell mixture. Fused cells were collected by centrifugation (100 ***g***, 10 min), resuspended in 250 ml medium supplemented with HAT (Sigma) and 20% 'myoclone' FCS (GIBCO) and pipetted into 10 24-well tissue culture plates (Falcon).

Hybridoma screening and cloning

After hybrid growth was visible, antibody-secreting hybridomas were selected by screening supernatants for immunoglobulins G, M and A using an ELISA adapted from a previously published method (Plater-Zyberk *et al.*, 1983) (supernatants from the patient B fusion were not screened for IgA). Supernatants were retested for immunoglobulin secretion on two further occasions at 10-day intervals and during this period HT supplement (Sigma) was substituted for HAT supplement in the culture medium. Hybridomas were cloned three times by limiting dilution in 96-well tissue culture plates (Falcon) using mouse macrophages as feeder cells. Not all patient A hybridomas were cloned and in these cases supernatants from original cultures were used for analyses.

Subclass and light chain determination

Monoclonal anti-light chain type and IgG subclass specific antibodies were used in ELISA and reverse passive haemagglutination assays to detect kappa or lambda light chain and IgG1, IgG2, IgG3 or IgG4 subclass in hybridoma supernatants.

In ELISA, neat supernatants were incubated in microtitre wells sensitized with monoclonal anti-kappa (6e1) or anti-lambda (C4) light chain for 2 h in a humidified incubator containing 5% CO_2 at 37°C. Bound IgM or IgG subclasses were revealed with horseradish peroxidase (HRP) conjugated sheep anti-human mu chain (The Binding Site, Birmingham, UK) or HRP-conjugated mouse monoclonal antibodies to IgG subclasses 1, 2, 3 and 4 (JL512, Gom2, ZG4 and RJ4, respectively) (produced in the Department of Immunology, Birmingham Medical School, Birmingham and available commercially from Oxoid Unipath, Bedford, UK).

In reverse passive haemagglutination, sheep red blood cells (SRBC) passively sensitized with monoclonal anti-light chains (C4, 6e1) or anti-IgG subclasses (JL512, Gom2, ZG4 and RJ4) were used (Ling, Bishop & Jefferis, 1977); 30 μl of 0·33% sensitized SRBC were added to serial double-folding dilutions of culture supernatants and agglutination results recorded after 1-h incubation at room temperature.

Isoelectric focusing

Neat supernatants (1 μl) and pI references standards (LKB Pharmacia, Bedfordshire, UK) were applied to the surface of an ultra-thin agarose isoelectric focusing gel equidistant from cathodic and anionic wicks. The gel was run in a LKB maxiphor apparatus and focused proteins transferred to nitrocellulose (Schleicher and Schuell, Anderman, UK). These procedures have been previously described (Williams *et al.*, 1986). Non-specific binding to nitrocellulose was blocked by incubation with a 2% casein solution (in PBS) and IgG electrofocusing patterns were visualized by incubation with HRP-conjugated goat anti-human IgG (ICN Biomedicals, Buckinghamshire, UK) diluted 1/2000 in a 0.5% casein solution (in PBS), washing with PBS and addition of diaminobenzidene, H_2O_2, cobalt and nickel as described by deBlas & Cherwinski (1983).

IgM-RF ELISA

Supernatants from all IgM-secreting hybridomas were screened for RF activity by measuring the binding to human IgG (Sigma), human IgG Fcγ fragments (Jackson Research Laboratories, West Grove, PA) or rabbit IgG (Sigma). Antigens were coated onto microtitre plates (Dynatech) at 10 μg/ml in PBS by overnight incubation at 4°C. Non-specific binding to plastic was blocked by incubation with 100 μl/well of a 2% casein solution (in PBS) for 1 h at 37°C. The casein solution was removed and supernatants or serial dilutions of reference sera (50 μl/well) were incubated for 1 h at 37°C. Wells were washed 6 times in a PBS containing 0·1% Tween 20 (Sigma) and bound IgM was revealed by incubation with alkaline phosphatase-conjugated goat anti-human IgM (Sigma) (1/1000 dilution in PBS/Tween)

(50 μl/well) for 1 h at 37°C. After washing as above *p*-nitrophenyl phosphate substrate (Sigma) was added (50 μl/well) and incubated for 1 h at 37°C. Optical density was read at 405 nm using a microplate autoreader (Biotek Instruments).

IgG-RF ELISA

Supernatants from IgG-secreting hybridomas were screened for RF activity in an ELISA by measuring the binding to Fc-Per, an IgG1-F paraprotein from a patient with H chain disease (Nik Jaafer *et al.*, 1983).

Microtitre ELISA plates (Flow) were sensitized with Fc-Per at 20 μg/ml in PBS by incubation at 37°C for 2 h. Unbound protein was removed by washing the plates three times with PBS containing 0.05% Tween 20. Supernatants were added undiluted (50 μl/well) and incubated for 2 h at 37°C in a humidified incubator with 5% CO_2. Bound IgG from culture supernatants were revealed with HRP-conjugated monoclonal antibody to the CH1 domain of human IgG (ZB8). The plates were then developed by the addition of peroxidase substrate (O-phenylene diamine) and results recorded as described above.

ELISA for determination of IgG subclass specificity of RFs

RF-positive supernatants were assayed for binding to IgG subclass proteins in ELISA. IgG paraproteins of different IgG subclasses, allotypes and light chain types were coated onto microtitre plates (Flow) at 20 μg/ml by incubation at 37°C for 2 h. Undiluted supernatants were added (50 μl/well) to sensitized wells and incubated at 37°C for 2 h in a humidified incubator with 5% CO_2. Bound IgM-RF was revealed with HRP-conjugated sheep anti-μ chain (The Binding Site) at 1/4000 in PBS/Tween. IgG-RF binding was revealed with the appropriate HRP-conjugated monoclonal anti-light chain antibody (C4 or 6e1, described above) with specificity to the light chain type opposite to that of the coating IgG paraprotein.

RESULTS

Fusion Efficiency

Small colonies were visible to the eye approximately 10 days after fusion. Fusion efficiency was calculated as the proportion of total B cells fused which gave rise to hybridomas (assessed by the number of wells with growing cells). Fusion efficiencies for patient A were $2{\cdot}7 \times 10^{-5}$ and for patient B $3{\cdot}125 \times 10^{-5}$.

Synovial B cell hybridomas

Seventeen IgG- and eight IgM-secreting hybridomas were derived from the synovial membrane of the seropositive RA patient (patient A) and nine IgG-secreting hybridomas were derived from the seronegative patient (patient B). These hybridomas have been maintained without loss of antibody secretion for 18 and 12 months, respectively, with periods of up to 4 months in continuous culture.

Table 1. Distribution of IgG subclass in 26 IgG secreting hybridomas derived from the synovial membrane of two patients with rheumatoid arthritis

Patient	IgG subclass secreted (no. of hybridomas)			
	IgG1	IgG2	IgG3	IgG4
A	17	0	0	0
B	8	1	0	0

Table 2. Distribution of light chain type in IgG and IgM antibodies secreted by hybridomas derived from rheumatoid synovial membranes

Source of hybridoma*	Class/ subclass	Light chain type		Kappa:lambda ratio
		kappa	lambda	
Patient A	IgG1	6	11	6:11
	IgM	6	2	3:1
Patient B	IgG1	4	4	
	IgG2	1	0	5:4

*Synovial membranes from two patients with rheumatoid arthritis

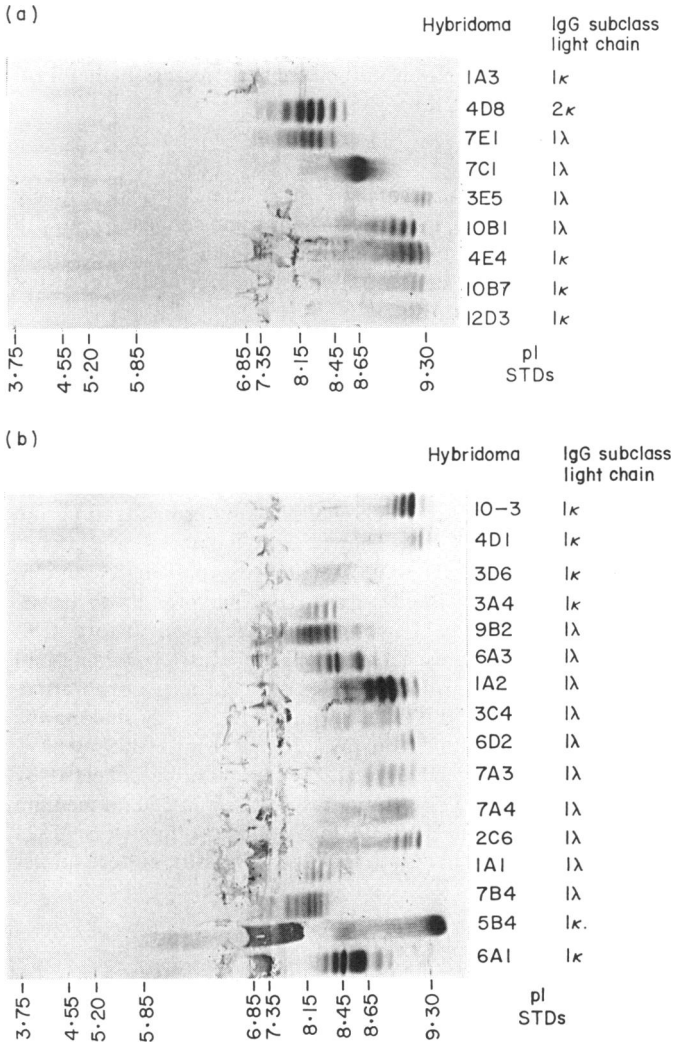

Fig. 1. Spectrotype of IgG antibodies secreted by (a) 16 hybridomas derived from the synovial membrane of patient A; (b) nine hybridomas derived from the synovial membrane of patient B. The corresponding subclass and light chain type of each hybridoma is indicated adjacent to the sample. Isoelectric points of a calibration kit are also indicated.

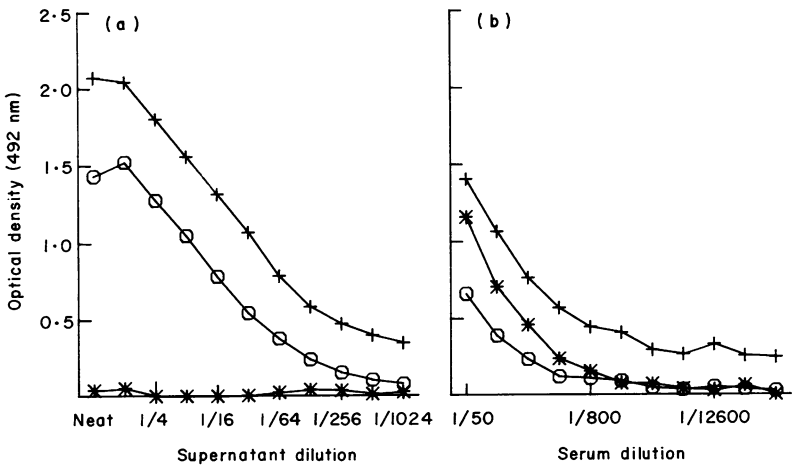

Fig. 2. Binding of IgM RF secreted by (a) hybridoma 2A2 derived from the synovium of RA patient A; and (b) IgM RF in the serum of patient A, to human IgG (+), human Fcγ (O) and rabbit IgG (*) in ELISA.

Table 3. Binding of monoclonal IgM rheumatoid factor (2A2) and IgG rheumatoid factor (7D4) to IgG subclass proteins in ELISA

		Patient A 2A2		Patient A 7D4	
		RF-ELISA		RF-ELISA	
IgG subclass	Gm allotypic marker	Antigen*	Binding	Antigen*	Binding
IgG1	f	Cri (λ)	+++	Rei (κ)	+
	zax	War (κ)	+++	War (κ)	+
IgG2	n (+)	Pea (κ)	±	Cla (κ)	+
	n (+)	Cam (κ)	++	Cam (κ)	+
IgG3	st	Goe (κ)	++	Goe (κ)	+
	b1	Ren (λ)	−	Ren (κ)	+
IgG4	(4a)	Car (λ)	+	Smi (κ)	±
	ND	Ree (κ)	+++	Ree (κ)	+

ELISA results are presented as +++ for optical density (OD) (492 nm) values > 1·0; ++ for OD values 0·5–1·0; + for OD values 0·5; and ± for OD values < 0·1 above the background level.

* The corresponding IgG paraprotein used as antigen in ELISA, light chain type is indicated in parentheses.

ND, not done.

IgG subclass distribution

All synovium-derived hybridomas secreted immunoglobulin of IgG1 subclass, with the exception of one from patient B which secreted IgG2 (Table 1).

Light chain distribution

Light chain analysis of the synovial B cell hybridomas from both patients is shown in Table 2. The kappa:lambda ratio of the IgG-secreting hybridomas from patients A and B is 6:11 and 5:4, respectively. In both cases (less noticeably in the patient B hybridomas) this demonstrates an over-representation of lambda light chain compared with the expected frequency of kappa:lambda 2:1 present in normal serum (Mannik & Kunkel, 1963; Fahey, 1963).

In contrast to the increased frequency of lambda light chain seen in the IgG-secreting hybridomas, the IgM-secreting hybridomas from patient A had a kappa: lambda ratio of 3:1.

Isoelectric focusing of hybridoma IgG antibodies

All the synovial membrane-derived IgG producing hybridomas secreted IgG in the cationic pI range 7·5–9·5 (Fig. 1). A proportion of the IgG spectrotypes showed similar banding patterns and the tight clustering of bands indicates the monoclonality of each hybridoma. Patient A hybridoma 7D4 was not focused due to low levels of IgG in the supernatant.

RF screening of hybridomas supernatants

One (patient A 2A2) of the eight IgM secreting hybridomas from patient A was producing RF. This antibody was an IgM kappa and bound in high titre to human IgG and human Fcγ fragments; however, it did not bind to rabbit IgG (Fig. 2). It was also shown to react with IgG subclasses 1, 2, 4 and IgG3 Goe of the Gm (st) allotype but not to other IgG3 proteins (Table 3). Low titre IgG RF activity was detected in one out of 17 (7D4) and one out of nine (4D8) supernatants of IgG-secreting hybridomas from patients A and B, respectively. Antibodies 7D4 and 4D8 were IgG1 lambda and IgG2 kappa, respectively; the former bound to all IgG subclasses (Table 3) (the latter was not tested in this assay).

DISCUSSION

Two fusions of mononuclear cells from rheumatoid synovial membranes (from one seropositive and one seronegative patient) resulted in a total of 26 IgG-secreting and eight IgM-secreting hybridomas. All eight IgM hybridomas were derived from the membrane of the seropositive RA patient. This is consistent with findings that IgM plasma cells are rarely seen in synovial membranes of seronegative patients (Munthe & Natvig, 1972; Revell & Mayston, 1986). We believe that this report is the first report of both IgG- and IgM-secreting hybridomas derived from synovium, and represents an advance in the investigation of B cell function in RA.

IgG1 was the predominant IgG subclass secreted by 100% (17/17) and 89% (8/9) of the hybridomas from two separate

fusions. This confirms the immunohistochemical findings of Mellbye *et al.* (1984) who reported that the majority of plasma cells in 15 synovia from seropositive and eight synovia from seronegative RA patients were secreting IgG1. However, bias towards secretion of other IgG subclass in rheumatoid synovia has also been documented (Munthe & Natvig, 1972; Hoffman *et al.*, 1982). In these cases IgG3 predominated; however, in one of these studies (Munthe & Natvig 1972) IgG1 or IgG2 restriction was demonstrated in four out of nine patients where > 50% of plasma cells secreted one subclass.

Another bias observed in our synovial-derived hybridomas was the predominance of lambda light chains in IgG antibodies. This was especially striking in the hybridomas from patient A (kappa:lambda 6:11). The kappa:lambda ratio in the hybridomas of patient B (5:4) did not show such a pronounced difference but when compared with the expected serum frequency this ratio again suggests an increased proportion of IgG immunoglobulins bearing lambda light chains.

Over-representation of particular IgG subclasses may reflect the nature of the antigen driving the immune response. IgG2 is the major subclass secreted in response to polysaccharide antigens (Riesen, Skarvil & Brown, 1976) and IgG1 and IgG3 are commonly involved in anti-viral immune responses (Skarvil, 1986; Sundquist, Linde & Wahren, 1984). It is also known that T cells regulate the expression of IgG subclass (Mongini, Stein & Paul, 1981; Teale, 1982; Mayumi *et al.*, 1983) probably via certain cytokines. For example, the T cell-derived lymphokine IL-4 preferentially enhances murine IgG1 and IgE secretion (Vitetta *et al.*, 1985; Coffman *et al.*, 1986). T cells are a major part of the inflammatory cell infiltrate in the rheumatoid joint and their interaction with B cells may result in amplification and over representation of a particular subclass.

Isoelectric focusing analysis demonstrates that all IgG secreted by the synovial derived hybridomas were cationic (Fig. 1). Since pathogenic antibodies in murine immune complex diseases are cationic (Ebling & Hahn, 1980; Yoshida, Yoshida & Lambert, 1985), this raises the possibility that potentially pathogenic antibodies may be represented in our panel. This possibility is further supported by the observation that cationic antibodies can bind to and penetrate articular cartilage (Zatarain-Rios & Mannik, 1987).

Overall the subclass, light chain and narrow pI range of the IgG antibodies suggest that activated IgG secreting B cells in the rheumatoid joint belong to a restricted population(s). It must also be considered, however, that fusion with the SPAZ-4 cell line could introduce a bias into the apparent IgG subclass and light chain repertoire by preferentially giving rise to IgG1 lambda-secreting hybridomas. Although IgM-secreting hybridomas were derived from one patient and showed kappa light chain predominance (six out of eight had kappa light chains), other human hybridomas secreting anti-viral or anti-bacterial antibodies resulting from fusions with the SPAZ-4 heteromyeloma and peripheral blood mononuclear cells taken from healthy individuals during vaccination also secreted IgG1 and had a kappa:lambda ratio of 5:8 (data not shown). Therefore, extrapolation of the IgG1 and lambda light chain bias in the synovial membrane derived hybridomas to the *in vivo* situation must be made with caution.

Specificity for the Fc fragment of human IgG was observed in one out of eight IgM and in one out of 17 IgG antibodies from one patient, and one out of nine IgG antibodies from the other patient. These findings are in agreement with studies of synovial membrane cell cultures (Wernick *et al.*, 1985) which showed that IgM-RF and IgG-RF synthesis represented 7·3% and 2·6% of total IgM and IgG produced; however, they are lower than the predicted frequency (up to 75%) of RF-secreting cells estimated from immunohistochemical examination of RA synovial membranes (Munthe & Natvig, 1972; Natvig & Munthe, 1975). Another report of B cell hybridomas derived from rheumatoid synovium (Randen *et al.*, 1989) illustrates that monoclonal RFs can be readily derived from this tissue. However, their methods included a 2–5 week stimulation with Epstein–Barr virus (EBV) prior to fusion and since EBV stimulation can induce RF *in vitro* (Slaughter *et al.*, 1978) the higher frequency of RF-secreting hybridomas from RA synovium is not therefore directly comparable with our results which are based on fusion of cells that have been activated in the synovium as part of the disease process.

The IgM-RF 2A2 derived from the synovial membrane of the seropositive patient has specificity for IgG subclasses 1, 2 and 4 (Table 3). This binding profile is identical to the Ga specificity of 'general' RFs found in the serum of rheumatoid patients, described initially by Allen & Kunkel (1966). Furthermore, 2A2 also bound to the IgG3 Goe of the G3m (st) allotype which differs from other IgG3 allotypes by having a histidine at residue 435 (like IgG1, 2 and 4) instead of an arginine. Monoclonal IgM RFs derived from RA patients bind to this IgG3 allotype (Jefferis, Nik Jaafer & Steinitz, 1984) and histidine at 435 appears necessary for the integrity of the Ga determinant.

RFs in the serum of RA patients (including the serum of the patient from whom 2A2 was derived) can also bind rabbit IgG. Rabbit IgG has been shown to inhibit the Ga specificity of IgM RFs, suggesting similar antigenic epitopes are shared by rabbit IgG and the Ga determinant (reviewed by Johnson & Faulk, 1976). It is interesting therefore that the 2A2 RF does not bind to rabbit IgG (Fig. 2) and is therefore recognizing an Fcγ epitope unique to human IgG. Studies using chemical modification of IgG (Hunneyball & Stanworth, 1976) have shown that autoantigenic determinants on human but not rabbit IgG involve tyrosine residues. The C-terminal half of the rabbit IgG Cγ2 domain lacks tyrosine residues which are present in the corresponding portion of human IgG. This suggests that determinant(s) unique to human IgG are present in this region. Therefore it is possible that the 2A2 monoclonal is recognizing a determinant that not only requires histidine at 435 (in the Cγ3 domain) but also involves other amino acids in the C-terminal half of the Cγ2 domain.

We report the use of a heteromyeloma human fusion partner to immortalise immunoglobulin secreting B cells from synovial membranes of two RA patients. Stable IgM- and IgG-secreting hybridomas were obtained. Restriction in the expression of subclass and light chain type and the narrow pI range of the IgG antibodies suggests selective mechanisms are involved in the expansion of B cell clones in the rheumatoid joint. Most of these monoclonal antibodies are of undetermined specificity, however, and therefore we cannot identify putative antigenic stimuli. Aside from specificity for IgG, binding to autoantigens such as collagen, cytoskeletal proteins and cardiolipin has been detected (manuscript in preparation) and suggests that the B cell response in RA has a polyclonal component. This type of B cell response is compatible with a cytokine-induced B cell prolifer-

ation and interleukin-6, a known polyclonal B cell activator, has been detected in rheumatoid joints (Hirano *et al.*, 1988).

Studies are now under way to determine fully the antibody specificity of the hybridomas, in order to provide further information regarding the nature of the factors driving the B cell response in RA.

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