Clin. exp. Immunol. (1977) 28, 197–203.

Rheumatoid factor plaque-forming cells in rheumatoid synovial tissue

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(*Received* 2 *November* 1976)

SUMMARY

Cells producing rheumatoid factor (RF) were readily detected *in vitro* by means of a haemolytic plaque assay system employing sheep erythrocytes (SRBC) sensitized with reduced and alkylated rabbit IgG anti-SRBC antibody as target cells. Rheumatoid factor-producing, plaque-forming cells (RF-PFC) were observed in all of the synovial tissue cell preparations from seropositive rheumatoid arthritis patients studied. The numbers of RF-PFC varied considerably without any direct correlation with serum titres of RF antibody activity. However, high numbers of RF-PFC were never found in patients with low rheumatoid factor titres whereas, high and low numbers of RF-PFC were found among the patients with high RF titres. Synovial tissue cell preparations from a group of seronegative patients, with only one exception, failed to exhibit RF-PFC.

INTRODUCTION

The rôle of anti-IgG antibodies, collectively termed as rheumatoid factors (RF), in the pathogenesis of rheumatoid arthritis has been a topic for active investigation for many years. Since the initial observations of intra-articular complement consumption clinically associated with seropositive rheumatoid arthritis (Hedberg, 1964; Hedberg, 1967; Pekin & Zvaifler, 1964) attention has been focused upon the inflamed joint and there are now several indications that RF may play an intrinsic part in the on-going inflammatory process within intra-articular spaces (Winchester, Kunkel & Agnello, 1971; Conn, McDuffie & Dyck, 1972; Munthe & Natvig, 1972; Zvaifler, 1973; Natvig, 1976; Johnson & Faulk; 1976). Local synthesis of immunoglobulins by rheumatoid synovial membrane has been demonstrated *in vitro* (Smiley, Sachs & Ziff, 1968) and various results indicate that rheumatoid factors are produced in plasma cells of rheumatoid tissue (Mellors *et al.*, 1959; McCormick, 1963; Nowoslawski & Brzosko, 1967; Munthe & Natvig, 1972). A variety of techniques have been employed for the detection of cells associated with RF (McCormick, 1963; Bonomo, Tursi & Gilardi; 1968; Smiley, Sachs & Ziff, 1968) but so far these have not facilitated either qualitative or quantitative evaluation of results. However, the recent reports of *in vitro* activation of complement by IgM-RF in a haemolytic assay system (Tanimoto *et al.*, 1975; Taylor-Upsahl *et al.*, 1977) have led us to develop a plaque assay technique for the detection of single cells producing IgM-RF. With the recent establishment of satisfactory procedures for the elution of cells from rheumatoid synovial tissue (Abrahamsen *et al.*, 1975), it was possible to study such cells directly in the plaque assay system. In this preliminary investigation, using sheep erythrocytes (SRBC) sensitized with reduced and alkylated rabbit IgG anti-SRBC antibody as target cells (Tanimoto *et al.*, 1975) in the liquid monolayer system of plaque assay (Cunningham & Szenberg, 1968), we have been able to detect a considerable number of cells producing RF in cell preparations of synovial tissue from patients with seropositive rheumatoid arthritis.

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MATERIALS AND METHODS

Elution of cells from synovial tissue. Nineteen patients with classical rheumatoid arthritis, four with juvenile rheumatoid arthritis and one with psoriatic arthritis were studied. Tissues were obtained at synovectomy performed in the joints and tendon sheaths of the fingers, wrist and foot and the elbow and knee joints.

Specimens were collected in Ca^{2+}- and Mg^{2+}-free Hanks's balanced salt solution (Grand Island Biological Co., U.S.A.) containing 2·5 i.u./ml heparin and antibiotics (50 i.u./ml penicillin and 50 μg/ml streptomycin) and were processed immediately. Cells were eluted from the specimens of synovial tissue following the method described in detail by Abrahamsen *et al.*, (1975). Briefly, the tissue was minced and then dissociated by treatment with 0·5 mg/ml crude collagenase and 0·15 mg/ml DNase (Collagenase Type 1 and DNase Type 1, Sigma Chemical Co., U.S.A.) at 37°C for 60 min followed by filtration through nylon mesh (single layer, pore size 200 μm, Nytal, Schweiz. Seidengazefabrik AG, Switzerland). The eluted cell suspension was centrifuged (150 *g* for 15 min) and the cells resuspended in tissue culture medium RPMI-1640 supplemented with 10% foetal calf serum (RPMI-1640 and foetal calf serum, FCS, Grand Island Biological Co., U.S.A.) and antibiotics. Cells were counted in a Bürker haemocytometer and viability was assessed by the trypan blue dye exclusion test. For differential counting of cells, small aliquots of cell suspension were run in a cytocentrifuge (Shandon Scientific Co. Ltd, U.K.), fixed in methanol and stained with May-Grunwald/Giemsa. Cell suspensions were adjusted to contain $1\text{–}10 \times 10^6$ viable cells per ml and were then ready for use in the liquid monolayer plaque assay system.

Sensitization of SRBC with reduced and alkylated rabbit anti-SRBC antibody. IgG fractions were isolated from two high-titred rabbit anti-SRBC sera (heat inactivated 56°C for 30 min) by ion-exchange chromatography on DEAE cellulose (DE-52, Whatman Ltd, U.K.) equilibrated with 0·015 M phosphate buffer, pH 7·6, as previously described (Taylor-Upsahl *et al.*, 1977). The IgG was reduced with 0·01 M dithiothreitol (Calbiochem., California, U.S.A.) at 37°C for 30 min, alkylated with 0·03 M iodoacetamide (Fluka AG Buchs SG, Switzerland) at room temperature for 20 min in the dark and finally, dialysed overnight against phosphate buffered saline (PBS), pH 7·2. Agglutination and lytic titres were determined as previously described (Taylor-Upsahl *et al.*, 1977).

SRBC (1%) suspended in 10 ml of appropriately diluted reduced and alkylated IgG haemolysin in PBS were incubated at 37°C for 30 min. The suspension was then centrifuged (1000 *g* for 5 min) and the SRBC washed once in PBS (10 ml) and finally resuspended in 0·4 ml PBS. This suspension of sensitized SRBC (approximately 20%) was then ready for use. Suspensions of unsensitized SRBC in PBS were employed as controls for the detection of cells producing natural antibody to SRBC.

Preliminary investigations in a 'pseudo-plaque' assay system. In order to avoid the use of the relatively limited supply of synovial tissue test material in the initial establishment of assay procedures, the ability to detect localised haemolysis of IgG-sensitized SRBC by RF diffusing from a single source was first investigated within an agarose gel system utilizing serum RF. The procedure was as follows. SRBC (0·2 ml), sensitized with varying dilutions (1/50–1/8000) of IgG haemolysin were mixed with 4 ml of molten 0·7% agarose in PBS (Indubiose A45, Agarose, L'Industrie Biologique Francaise, France) and the mixtures poured into plastic Petri dishes (90 × 15 mm. Heger Plastics A/S, Norway). A series of wells, 2·5 mm diameter, were then cut out of the agarose and filled with various RF-positive and RF-negative sera which had been heat inactivated (56°C for 30 min) and absorbed with 20% SRBC (4°C for 60 min). Non-reduced and alkylated rabbit IgG haemolysin and PBS were employed on each plate as positive and negative controls of haemolysis, respectively. In addition, control plates were prepared with unsensitized SRBC in agarose. The plates were incubated at 37°C for 60 min followed by addition of 3 ml of a 1/10 dilution of fresh guinea pig serum as complement source and incubation for a further 30 min. Guinea-pig serum (National Institute of Public Health, Norway), in 10-ml aliquots, was always absorbed with 20% SRBC before use in any assay system. The plates were then drained of excess liquid and stored at 4°C overnight. Haemolysis was assessed macroscopically.

Levels of reduced and alkylated IgG haemolysin determined as optimal for sensitization of SRBC in the agarose gel technique were then investigated in the liquid monolayer plaque assay system.

Liquid monolayer plaque assay. The liquid monolayer system of plaque assay (Cunningham, 1965) was selected for these studies as this system has, in our hands, consistently proved to be more sensitive than the Jerne and Nordin agar gel technique (1963) for the detection of plaque-forming cells (PFC).

Slide chambers were assembled as suggested by Cunningham & Szenberg (1968) employing three pieces of double-sided adhesive tape (Scotch Brand Tapes Three M's, Minnesota, U.S.A.) to divide the slides into approximately equal areas. Forty microlitres of tissue culture medium TC-199 (Gibco Bio. Cult. Ltd., U.K.) containing 0·22% $NaHCO_3$ and supplemented with 5% FCS (heat inactivated 56°C for 30 min) was pipetted into small glass tubes (10 × 80 mm) and to this was added 20 μl of a 1/2·5 dilution of fresh guinea-pig serum in TC-199 and 20 μl of IgG-sensitized SRBC. Synovial tissue cell suspension, 100 μl, was then added to the tubes and the contents mixed thoroughly. Slide chambers were filled with these mixtures, sealed with paraffin wax and incubated in a horizontal position at 37°C for 90 min. The numbers of plaques were counted macroscopically. Results are generally expressed as number of rheumatoid factor-producing, plaque-forming cells (RF-PFC) per 10^6 viable cells examined. Varying dilutions of initial cell suspension were tested each time in order to investigate a range of cell concentrations and facilitate counting of plaques. Unsensitized SRBC were treated in exactly the same manner as described for IgG-sensitized SRBC. In addition, control slides containing IgG-sensitized SRBC, TC-199 and complement in the absence of synovial test cells were always included as a further check on the inability of the levels of

reduced and alkylated IgG haemolysin used to sensitize the SRBC to fix complement and cause subsequent lysis of SRBC under assay conditions.

Estimation of rheumatoid factor titre. Sera were tested for RF antibody activity in a slide latex fixation test (Baxter Laboratories, Thetford, U.K.) and RF titres were obtained using a slightly modified Waaler-Rose haemagglutination system employing human 0 erythrocytes coated with rabbit antibody (Mellbye & Natvig, 1970). Of the twenty-four patients studied, thirteen were seropositive and eleven were seronegative.

RESULTS

Studies employing the agarose gel 'pseudo-plaque' assay

Discrete zones of lysis of IgG-sensitized SRBC were observed around wells containing RF-positive sera. The size of the haemolytic zones varied according to RF titre of the serum and it was apparent that the clarity of the zones was dependent upon the levels of reduced and alkylated IgG haemolysin used to sensitize the SRBC. Dilutions of 1/1600 and 1/400 of the two IgG haemolysin preparations with haemagglutination titres of 4096 and 1024, respectively, were found to be optimal for initial sensitization of SRBC facilitating clear demonstration of localized haemolysis by RF. With higher dilutions of IgG haemolysin the haemolytic zones were less distinct whereas, lower dilutions resulted in significant levels of background agglutination and lysis of SRBC. This background haemolysis was due to the failure of reduction and alkylation procedures to totally eliminate the complement-fixing ability of the rabbit IgG, haemolytic titres of the two haemolysin preparations post reduction and alkylation being 1024 and 128, respectively. A pro-zone of inhibition of haemolysis of optimally sensitized SRBC was detected with some of the RF-positive sera. Lysis of IgG-sensitized SRBC was not observed with any of the RF-negative sera studied and, on control plates, neither RF-positive nor RF-negative sera exhibited lysis of unsensitized SRBC.

Studies employing the liquid monolayer plaque assay

The levels of reduced and alkylated IgG haemolysin determined as optimal for sensitization of SRBC within the agarose gel system also proved to be optimal when tested in the liquid monolayer plaque assay system. Results obtained with either of the haemolysin preparations at optimal concentration were directly comparable.

Haemolytic plaques were observed with each of the thirteen synovial tissue cell preparations from seropositive patients studies whereas, only one of the eleven cell preparations from seronegative patients exhibited plaque formation. Plaques were not detected in control slides containing either unsensitized SRBC and test cells or IgG-sensitized SRBC in the absence of synovial tissue test cells. The plaques varied in size from 0·5–2·0 mm in diameter and were totally clear allowing microscopic examination and, in most instances, detection of a single RF-PFC positioned more or less centrally within each haemolytic zone.

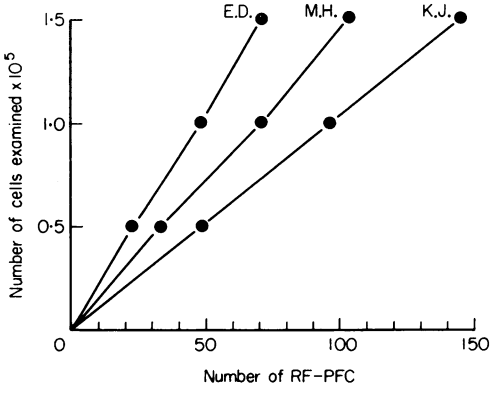

FIG. 1. Relationship between the number of RF-PFC observed and the number of synovial tissue cells examined in three seropositive patients with rheumatoid arthritis.

TABLE 1. Detection of RF-PFC in cell suspensions eluted from synovial tissue. Of the twenty-four patients studied, one had psoriatic arthritis (P), four had juvenile rheumatoid arthritis (J) and nineteen had classical rheumatoid arthritis. Results are expressed as number of RF-PFC per 10^6 viable cells examined (± s.e.)

Patient	Age	Sex	Operated joint	RF activity in serum		Number of RF-PFC/10^6 synovial tissue cells
				Latex	Waaler-Rose	
M.B. (J)	10	F	Fingers	0	< 16	0
M.A. (J)	15	F	Toes	0	< 16	0
T.Ø. (J)	15	M	Wrist	0	< 16	0
I.F. (J)	13	F	Wrist	0	< 16	0
T.L. (P)	19	F	Knee	0	< 16	0
O.P.	33	M	Wrist	0	< 16	0
P.B.	44	M	Wrist	0	< 16	0
A.F.	56	M	Knee	0	< 16	0
K.E.	56	F	Wrist	0	< 16	0
Å.L.	65	F	Wrist	0	< 16	0
E.B.	47	F	Knee	0	< 16	130 (± 7)
M.H.	50	F	Fingers	+1	64	682 (± 14)
K.J.	64	M	Wrist	+2	64	482 (± 39)
M.H.	54	F	Wrist	+2	64	136 (± 15)
K.J.	32	M	Elbow	+3	128	964 (± 55)
R.H.	56	F	Wrist	+3	256	11 (± 0·6)
E.D.	62	F	Knee	+3	512	454 (± 18)
M.N.	53	F	Fingers	+3	512	224 (± 41)
O.N.	50	M	Elbow	+3	512	3958 (± 377)
E.K.	58	F	Fingers	+3	512	138 (± 13)
L.S.	48	F	Wrist	+4	1024	432 (± 86)
E.T.	63	F	Knee	+4	1024	2353 *
B.J.	73	M	Knee	+4	1024	17,245 (± 102)
H.D.	66	M	(a)Right wrist	+4	1024	1687*
			(b)Left wrist			2123 (± 246)

* Insufficient cells for replicate studies. Note that each tissue from seropositive patients exhibits RF-PFC whereas only one of the tissues from seronegative patients contains demonstrable RF-PFC.

A range of cell concentrations of each synovial tissue cell preparation was studied and the numbers of RF-PFC were seen to increase in proportion to increasing numbers of cells examined (Fig. 1). In cell preparations containing high numbers of RF-PFC, total lysis of IgG-sensitized SRBC was frequently observed with the highest concentrations of cells tested and, where sufficient cells were available, the assay was repeated using increased dilutions of original cell suspension.

The patients studied covered a wide range of age groups and latex and Waaler-Rose titres of RF antibody activity as can be seen from Table 1. The four cases of juvenile rheumatoid arthritis and one case of psoriatic arthritis, included among the seronegative patients, each failed to exhibit RF-PFC (Table 1). Generally, the total number of RF-PFC varied considerably among seropositive patients apparently regardless of serum titres of RF activity (Fig. 2). However, high numbers of RF-PFC were never found in patients with low RF titres whereas, high and low numbers of RF-PFC were seen among the patients with high RF titres (Fig. 2).

Differential counts of the cell preparations from synovial tissue showed a general predominance of lymphocytes and macrophage-like cells. Cells exhibiting relatively strong basophilic staining, some of which had the appearance of plasma cells, are grouped among the unclassified cells (Table 2). Preliminary studies of RF-PFC-positive cell preparations retested in the plaque assay system following removal of adherent cells and separation of lymphocytes by Isopaque–Ficoll gradient centrifugation (Abrahamsen

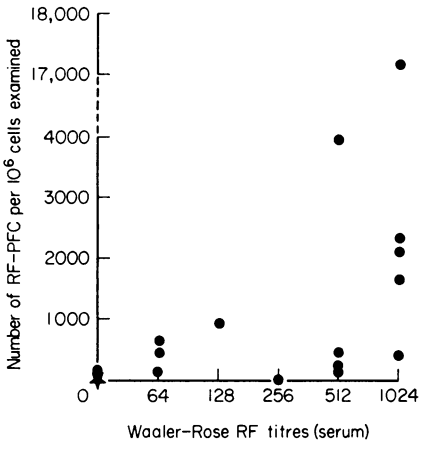

FIG. 2. A comparison of RF titres in serum and number of RF-PFC detected in cell preparations from synovial tissue. (✱) Cell preparations from ten seronegative patients including one with psoriatic arthritis, four with juvenile rheumatoid arthritis and five with rheumatoid arthritis, not exhibiting RF-PFC. (●) Individual observations of RF-PFC in cell preparations from one seronegative patient and each of thirteen seropositive patients with rheumatoid arthritis.

TABLE 2. Differential counts of synovial tissue cell suspensions tested in the liquid monolayer plaque assay system

	K.E.	E.B.	M.H.	E.D.	K.J.	M.H.	H.D.	O.N.	B.J.
Lymphocytes	58	55	58	73	27	46	68	45	59
Macrophage-like cells*	39	41	36	24	65	34	23	38	27
Neutrophils	1	0	0	2	1	2	0	4	0
Unclassified cells†	2	3	5	1	6	16	9	12	13
Dead cells‡	0	1	1	0	1	2	0	1	1
RF-PFC/10^6 viable cells	0	130	136	454	482	682	2123	2958	17245

All figures above the broken line indicate percentages of cells. At least 200 cells from each patient were counted.

* May contain type A synovial cells.

† May contain plasma cells and type B synovial cells.

‡ Cells with pyknotic nuclei.

et al., 1975) have indicated marked losses e.g. 73–100% loss, in the number of RF-PFC. However, this observation requires further and more detailed study to determine at which stage in the separation procedures loss of RF-PFC occurs.

DISCUSSION

The complement-fixing ability of rheumatoid factor, RF, can clearly be used as a means of detection of cells producing RF in a plaque assay system. The ability of IgM-RF to activate complement was recently demonstrated by Tanimoto *et al.* (1975) in a haemolytic assay system employing SRBC sensitized with reduced and alkylated rabbit IgG anti-SRBC antibody as target cells. This approach utilizes the observation that reduction and alkylation of rabbit antibody markedly diminishes its complement-fixing ability (Wiedermann, Miescher & Franklin, 1963; Schur & Christian, 1964) while retaining antigen-binding activity and reactivity with RF (Zvaifler & Schur, 1968).

In the present study, such IgG-sensitized SRBC have successfully been employed as target cells in a 'pseudo-plaque' assay system for the detection of RF in sera and in a liquid monolayer plaque assay system for the detection of RF-producing, plaque-forming cells, RF-PFC. Observations made with the 'pseudo-plaque' assay system were relatively straightforward, RF activity being detected in sera from all

of the seropositive patients tested but not in any of the sera from seronegative patients. A prozone of inhibition of haemolysis, observed with several of these RF-positive sera, was likely due to the presence in the sera of RF-binding immunoglobulin material, the inhibitory effects of which have previously been encountered in the haemolytic assay of complement fixation by RF (Taylor-Upsahl *et al.*, 1977). The observations made with the liquid monolayer plaque assay system were not, however, quite as clearly defined, RF-PFC being detected in one of the cell suspensions from a seronegative patient and the numbers of RF-PFC seen in seropositive donor cell preparations varying considerably regardless of serum titres of RF activity.

The general lack of direct correlation between the local synthesis of RF in affected tissues, as assessed by numbers of RF-PFC, and the titres of RF activity in peripheral circulation may well be reflecting differences among patients in the rates of removal of free RF from these areas dependent upon such factors as amount of IgG complexes present (Hannestad, 1967; Munthe & Natvig, 1972). Differences in levels of production of RF in different joints of the same patient could also contribute to these variations. Thus, despite significant production of RF at a single site, the RF might be blocked locally by IgG complexes and consequently go undetected or detected in only low titres in the serum. Conversely, RF observed in high titres in the serum may come from sites other than those studied where levels of RF production appeared low.

The plaque assay system described, as with the haemagglutination systems for estimation of RF titre (Turner, 1967) and the haemolytic assay of complement fixation by RF (Taylor-Upsahl *et al.*, 1977) is likely detecting only IgM-RF. Immunofluorescent studies on rheumatoid synovial membrane have previously demonstrated that the free RF detectable within plasma cells of seropositive patients is almost entirely IgM-RF, whereas, free RF of any one immunoglobulin class is rarely observed with tissues of seronegative patients (McCormick, 1963; Munthe & Natvig, 1972). IgG-RF-associated plasma cells are known to be present in synovial tissues of both seropositive and seronegative patients (Munthe & Natvig, 1972) and the complement-fixing ability of IgG-RF has been demonstrated (Bianco, Dobkin & Schur, 1974). However, IgG-RF activity is generally thought to be blocked intra- and extra-cellularly due to the formation of IgG-IgG-RF complexes (Munthe & Natvig, 1972). Our positive findings of RF-PFC in synovial tissues from seropositive patients and generally negative results obtained with tissues from seronegative patients are consistent with a lack of free IgG-RF with detectable complement-fixing ability.

It may be possible, by inclusion of various procedures such as pepsin digestion in the case of IgG-RF (Munthe & Natvig, 1972; Pope, Teller & Mannik, 1975) and the use of monospecific immunoglobulins (Wortis, Dresser & Anderson, 1969; Plotz, Talal & Asofsky, 1968) in an open plaque assay system, to detect local synthesis of RF of immunoglobulin classes other than IgM and, in addition, to demonstrate the presence, if any, of non-complement-fixing IgM-RF (Tanimoto *et al.*, 1975). In conclusion, local synthesis of rheumatoid factor by cells present in cell preparations eluted from rheumatoid synovial tissue of seropositive patients is readily detectable within a haemolytic plaque assay system. This observation should provide an invaluable tool for the characterization of cells producing rheumatoid factor and for future wide ranging comparative studies of rheumatoid factor activity at site of production.

The authors wish to thank Miss Kari Ulrichsen for skilled technical assistance. M.M.T.-U. would also like to thank The Royal Society, London, for the award of a European Science Exchange Fellowship.

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