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Research Article

Lymphocytes secreting anti-IgC antibodies, rheumatoid factors (RF), can be detected in the peripheral bloods, synovial fluids, and bone marrows of patients with seropositive rheumatoid arthritis by using a direct plaque-forming cell (PFC) assay with sheep erythrocytes sensitized with reduced and alkylated rabbit IgG hemolysin. The autospecific nature of the RF produced by RF-PFC was indicated by inhibition studies in which the order of patency was human IgG greater than rabbit IgG greater than bovine IgG. In metabolic studies puromycin, cycloheximide, and venblastine suppressed RF-PFC. Cyclic AMP and cyclic GMP were without effect. A need was recognized for using full tissue culture media during the cell separation and plaquing procedures to optimize detection of the RF-PFC. RF-PFC may appear in the blood of patients intermittently despite their continuing presence in the bone marrow. They have been found in the peripheral blood, especially during acutely exacerbating polyarticular synovitis, generalized vasculities, or generally active, aggressive disease. RF-PFC were found in synovial effusions of new or recrduescent acute synovitis. RF-PFC were observed to disappear from the peripheral circulation and the bone marrow during therapy with cytotoxic drugs. The data are consistent with the hypothesis that the appearance of RF-PFC in the peripheral blood represents an anamnestic response to transiently appearing antigen. The nature of the antigen is not specified. The bone marrow may be […]

Rheumatoid Factor-Producing Cells Detected by Direct Hemolytic Plaque Assay

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ABSTRACT Lymphocytes secreting anti-IgG antibodies, rheumatoid factors (RF), can be detected in the peripheral bloods, synovial fluids, and bone marrows of patients with seropositive rheumatoid arthritis by using a direct plaque-forming cell (PFC) assay with sheep erythrocytes sensitized with reduced and alkylated rabbit IgG hemolysin. The autospecific nature of the RF produced by RF-PFC was indicated by inhibition studies in which the order of potency was human IgG > rabbit IgG > bovine IgG. In metabolic studies puromycin, cycloheximide, and vinblastine suppressed RF-PFC. Cyclic AMP and cyclic GMP were without effect. A need was recognized for using full tissue culture media during the cell separation and plaquing procedures to optimize detection of the RF-PFC.

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INTRODUCTION

Several methods have been reported to detect rheumatoid factor (RF) [1] in, or associated with, lymphoid cells. These have included staining with fluorescent-labeled aggregated human IgG (1–4), mixed agglutination in fixed tissues (5), and production of rosettes with sensitized erythrocytes mixed with lymphocytes (6, 7). None of these procedures, however, assesses RF production. Culture of mixed cell suspensions in the presence of radio-labeled amino acids (8) has indicated RF production by yielding radiolabeled RF, but the numbers or types of cells involved in the production was not revealed by this method.

Some of our recent observations brought forth the possibility that we could detect RF production by single cells. We have shown (9) that: (*a*) IgM RF fixes and activates guinea pig and human complement via the classical complement pathway; (*b*) IgM RF prepares sheep erythrocytes coated with reduced and alkylated rabbit IgG hemolysin for lysis by complement; and (*c*) the percentage of hemolysis is directly proportional to RF agglutination titer.

These observations led us to attempt to use the direct hemolytic plaque assay described by Jerne et al. (10) to identify lymphoid cells producing RF. With sheep erythrocytes (SRC) sensitized with reduced and alkylated rabbit IgG hemolysin, we have examined lymphocyte preparations from peripheral blood, synovial fluid, and bone marrow and found cells secreting RF plaque-forming cells (PFC) in all three sites, but in highest numbers in the bone marrow.

The presence of RF-PFC in the peripheral blood correlates with active, exacerbating, or aggressive disease.

METHODS

Subjects. The patient populations studied included an initial group of 72 patients with seropositive rheumatoid

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[1] *Abbreviations used in this paper:* BM, bone marrow; BSS, balanced salt solution; FBS, fetal bovine serum; LFT, latex fixation test; PBL, peripheral blood lymphocytes; PFC, plaque-forming cell; RA, rheumatoid arthritis; RF, rheumatoid factor; SCAT, sensitized sheep cell agglutination test; SRC, sheep erythrocytes.

arthritis (RA) randomly selected from our clinics, 14 with seronegative RA, 14 non-RA patients with a variety of rheumatic diseases, and 24 normal individuals who were RF negative. The patients with RA were all classical or definite RA according to the American Rheumatism Association criteria (11). A second series included 16 seropositive RA patients most of whom were on a drug trial with Naprosyn (Syntex Laboratories, Inc., Palo Alto, Calif.). They were selected as a group because they had generally mild-to-moderate disease and were not on corticosteroids. A third group consisted of 18 patients selected for study because of the clinical impression of more active or aggressive disease in them.

Most patients were examined in our ambulatory clinics. Some were hospitalized in our Clinical Research Center for more prolonged and intensive investigations. The patients on the drug study were followed in the ambulatory facility of our Clinical Research Center, where more detailed clinical observation could be made than in our regular clinics. All patients were fully informed of the nature of the studies undertaken and gave willing consent.

Clinical characteristics. RA patients with any evidence of generalized vasculitis were assigned to the vasculitis group. Manifestations of vasculitis ranged from nail fold infarcts to mononeuritis multiplex and typical vasculitic cutaneous ulcerations. In this report no distinction is made between patients with minimal and severe vasculitis.

Patients were regarded as having an acute flare if they had acute synovitis of less than a 2-wk duration. A joint already exhibiting synovial hypertrophy and effusion was considered the site of recrudescent acute synovitis only in the event of sudden increase in joint volume, tenderness, pain, erythema, or heat. Patients with two or more acutely inflamed joints were considered to have polyarticular synovitis.

All patients who were seen in our Clinical Research Center were evaluated serially with the American Rheumatism Association Database (12) as the form of clinical assessment.

Preparation of lymphocytes. Lymphocytes from peripheral blood and synovial fluid were generally purified by differential centrifugation on Ficoll-Hypaque (13), or occasionally by dextran sedimentation with iron lysine (14, 15). Preparations from peripheral blood consisted of cells appearing morphologically greater than 92% viable small lymphocytes. Lesser degrees of purity of the lymphocytes were encountered with some preparations from synovial effusions.

Bone marrow was aspirated from the posterior iliac crest, or in some instances from the sternum, 5 ml being taken into a plastic syringe containing 10 mg of heparin. The cells were washed in buffer containing 10% fetal bovine serum (FBS), and then submitted to hypotonic lysis of the erythrocytes for 10 min at 37°C in 0.83% NH_4Cl. They were then washed twice further in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) with 10% FBS and used for RF-PFC.

Sensitized sheep cells. Rabbit anti-SRC hemolysin purchased from Baltimore Biological Laboratories (Division of BioQuest, Cockeysville, Md.) was separated into IgM and IgG fractions by Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gel filtration. IgG hemolysin was reduced and alkylated and used to sensitize SRC as reported previously (9).

Measurement of RF activity. The RF activity of sera and synovial effusions was measured by the slide latex fixation test (LFT, RA test, Hyland Laboratories, Los Angeles, Calif.) and by the sensitized sheep cell agglutination tests (SCAT) (16). The latter was carried out by settling patterns in microtiter plates.

Source of complement. Lyophilized guinea pig serum (Hyland Laboratories) was reconstituted with the diluent provided. For some experiments natural antibody to SRC was absorbed by repeated incubation at 0°C with packed SRC as reported previously (9).

PFC assay for RF production. 0.5 ml of 0.5% agarose at 44°C (agarose A-37 Indubiose R, L'Industrie Biologique, Française, S. A., Gennevilliers, France) was mixed with 0.1 ml of lymphocyte suspension containing $1–5 \times 10^6$ lymphocytes and 0.05 ml of a 6.7% suspension of SRC coated with reduced and alkylated rabbit IgG hemolysin. The mixture was then promptly poured onto precoated microscope slides. The slides were incubated at 37°C for 90 min in a moist chamber, then immersed in a 1:10 dilution of guinea pig complement and incubated an additional 90 min at 37°C. Preabsorption of the guinea pig complement to remove natural antibody to SRC did not prove to be necessary since there was no significant background lysis of the unsensitized SRC controls. RF-PFC were enumerated under magnification. Similar cell suspensions employing nonsensitized erythrocytes were examined as controls to detect any lymphocytes synthesizing natural antibody to SRC.[2] Control PFC were subtracted from PFC with sensitized erythrocytes and the results were expressed as RF-PFC/10^6 lymphocytes. Generally, four replicate determinations were made for each preparation.

Metabolic inhibition. Puromycin, cycloheximide, and dibutyryl cyclic AMP were obtained from Calbiochem, San Diego, Calif. 8-bromo cyclic GMP was obtained from Sigma Chemical Co., St. Louis, Mo. Vinblastine was obtained from Eli Lilly and Co., Indianapolis, Ind. Inhibitors were added to 0.5-ml aliquots of melted agar (*vide supra*) in 10-μl volumes just before the lymphocytes and sensitized erythrocytes. Thereafter, the preparations were poured and subsequently handled in the usual fashion.

Preparation of data. Statistical comparisons were made by the Student's t test on log transformation. The exponentiated mean±SEM is used throughout the paper.

RESULTS

Typical direct hemolytic plaques are produced in our RF-PFC assay. The plaques vary in size from pinpoint to 0.3–0.5 mm. At the numbers of leukocytes used per slide, each plaque contained multiple leukocytes microscopically, so that the single central lymphoid cell, the putative antibody secreting cell, could not be distinguished with certainty. Leukocyte clustering was sometimes seen, more frequently in preparations containing bone marrow or synovial fluid cells than in the peripheral blood cells. Most clusters in such preparations were not surrounded by hemolytic zones, but when present the zones generally were larger than the zones surrounding single cells, suggesting multiple RF-PFC in such clusters.

[2] This control was almost always negative in the peripheral blood, even at 5×10^6 lymphocytes per slide. In bone marrow preparations, 1–3 PFC/10^6 nucleated cells were occasionally seen with nonsensitized SRC.

Immunological studies. The RF-PFC were complement dependent, since leaving complement out of the assay resulted in no plaques, and introduction of complement earlier during the assay, i.e., at the time of mixing of the cells with the agarose, resulted in earlier appearance of the plaques.

The specificity of the RF-PFC assay has been investigated. Since rabbit rather than human IgG hemolysin was used to sensitize the sheep cells in the assay, the question had to be answered whether the PFC were due to heterospecific rather than autospecific antibodies. To answer this question, inhibition studies were carried out, in which human, rabbit, or bovine IgG was incorporated in varying doses in the agar (Fig. 1). Three patients' cells were examined (Ta, So, and Ra). Both bone marrow (BM) and peripheral blood lymphocytes (PBL) were used as sources for the RF-PFC. Human IgG regularly provided better inhibition than rabbit IgG. Bovine IgG was least effective. Therefore, the RF (anti-IgG) detected in the PFC assay can be properly regarded as autoantibody. There was no doubt about the specificity of the inhibition of RF-PFC, since the RF-PFC were not inhibited in high concentrations of other serum proteins. For instance, there was no inhibition in 10% FBS.

A surprise was that human IgG actually had greater efficacy than rabbit IgG in the RF-PFC inhibition, since the common experience in assaying the peripheral

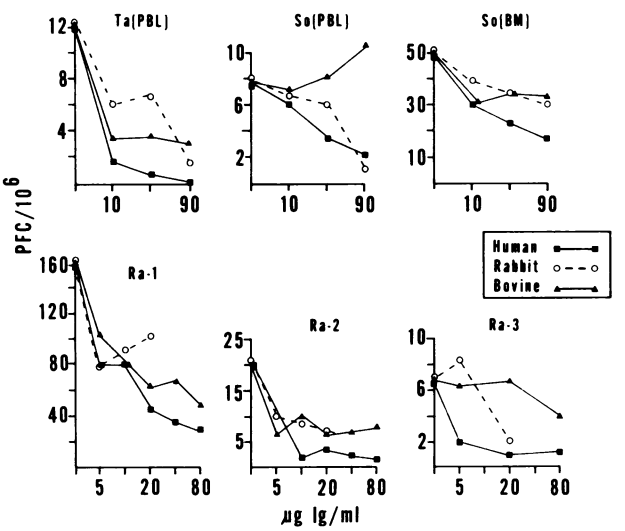

FIGURE 1 Inhibition of RF-PFC by various species of animal IgG. The indicated concentrations of IgG were incorporated in the agar in BSS along with the lymphoid cell population examined. Three patients, Ta, So, and Ra, were studied. For patient So, results with both PBL and BM are shown. For patient Ra, results are shown for the BM at three different times during the course of immunosuppressive therapy, during which a reducing number of RF-PFC were seen.

TABLE I
Inhibition of Serum RF-SCAT by Addition of IgG of Various Species

Patient		Rabbit	Human	Bovine
So.	1/12,000	4*	2*	1*
Ra.	1/5,000	8	4	1
Ta.	1/20,000	3	2	1

* Reduction of titer (number of tubes) by addition of 160 μg IgG. The IgG was added to 1 ml of 1/50 (So. and Ta.) or undiluted (Ra.) serum and the agglutinating end point compared to that of the original serum.

blood serum for RF with the SCAT has been that rabbit IgG is a better inhibitor than human IgG (17, 18). The SCAT assay, like the RF-PFC assay, used SRC sensitized with rabbit IgG as the indicator cell. Thus the RF at its source (the RF-PFC assay) appeared to be different from RF in the peripheral serum (the SCAT assay), as will be discussed later.

To rule out that the three patients we studied were atypical, we performed inhibition assays on the SCAT reactions of the RF in these patients' sera taken at the time of the RF-PFC assays. The results are shown in Table I. The peripheral blood serum RF activities were inhibited best by rabbit IgG, less well by human IgG, and little or none by bovine IgG, just as had been described for RF in the serum previously (17, 18).

The RF-PFC detected in our assay have been of the direct variety and therefore are assumed to represent IgM complement-fixing RF (10). Attempts have been made to detect other varieties of RF by indirect means. We have added an antihuman IgG serum along with complement at the second incubation step in the assay (10) in the attempt to detect IgG RF. This has generally not yielded additional PFC and sometimes has inhibited because of the IgG present in the antiserum. Antihuman IgM antibody has been added in the attempt to detect noncomplement-fixing IgM RF-PFC, but no such PFC have been found.

Since our assay detects RF-PFC only by the cross-reaction of the RF with rabbit IgG, attempts have been made to develop a cell coated with human IgG that can be used in the assay. The Ripley anti-CD antibody (19) has been tried, but it becomes strongly agglutinating when reduced and alkylated, rendering it unsuited to our test. Coating of sheep cells with human IgG by means of chromic chloride has not yielded PFC. Further efforts in these directions, however, are needed.

We carried out studies to determine whether the RF detected in our RF-PFC assay could have been due to RF adsorbed to leukocytes and carried over by them into the agar phase of the assay, to be released there and recognized as hemolytic plaques. To test this we

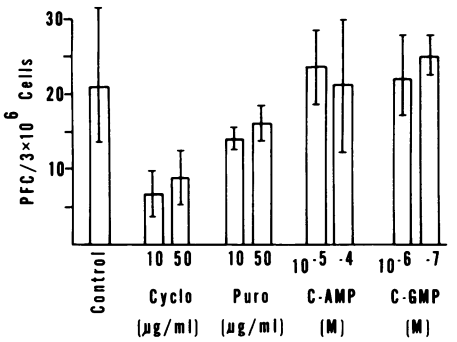

FIGURE 2 Effects on RF-PFC of inhibitors of protein synthesis and cyclic nucleotides. Reduction of numbers of RF-PFC by inhibition of protein synthesis with cycloheximide (cyclo) and puromycin (puro). Lack of effect of cyclic (c) nucleotides. The inhibitors were added to the agarose before the addition of the lymphoid cells. Subsequent incubation at 37°C was carried out in standard fashion (see Methods).

used peripheral blood leukocytes both from normal donors and from patients with active seronegative RA and a BM preparation from a normal volunteer. None of these cells developed detectable RF-PFC in 3×10^6 lymphocytes after exposure to the sera of two patients with RF titers of 5,120, who had themselves exhibited 49 and 18 RF-PFC/3×10^6 lymphocytes at the times that their sera were collected. This failure was true even when the sera and leukocytes were preincubated overnight at 4°C.

Metabolic studies. Efforts to determine some of the metabolic requirements for RF-PFC were carried out. Dependence upon protein synthesis was tested by addition of cycloheximide or puromycin to the agar; influence of the cyclic nucleotides was tested with dibutyryl cyclic AMP and 8-bromo cyclic GMP; and importance

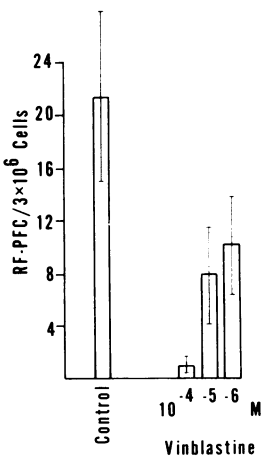

FIGURE 3 Inhibition of RF-PFC by vinblastine. The varying concentrations of vinblastine were added to the agarose before the addition of blood lymphocytes. Subsequent incubation at 37°C was carried out in standard fashion.

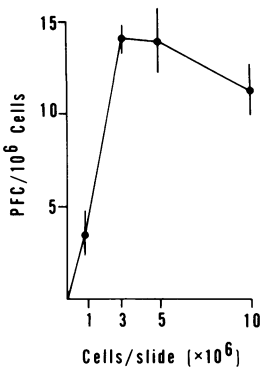

FIGURE 4 Inhibition of RF-PFC at high cell concentrations. The indicated numbers of peripheral blood leukocytes were added to 0.5 ml of agarose in BSS and incubated in standard fashion at 37°C.

of microtubular or membrane integrity was tested with vinblastine (20). Of many experiments performed, those shown in Figs. 2 and 3 are typical. Cycloheximide and puromycin generally reduced the number of RF-PFC, but not below about 35% of the starting number. Inhibition was effected as well by 10 μg as by 50 μg of each drug. Dibutyryl cyclic AMP and 8-bromo cyclic GMP have had no effect. Vinblastine reduced the PFC in several experiments, and occasionally at 0.1 mM essentially to zero. In studies not shown, colchicine had essentially no inhibiting effect when used by itself, but inhibited modestly in the presence of cytochalasin B. Cytochalasin B by itself also generally failed to inhibit.

These observations have been taken to mean: (*a*) only a minority of the RF-PFC have enough stored RF for plaque formation in the absence of continued protein synthesis; (*b*) the cyclic nucleotides probably do not modulate significantly the synthesis or release of the RF; and (*c*) the release depends upon intact microtubular or membrane structures.

During the course of these experiments, attempts were made to use increasing numbers of lymphocytes in the agar to increase the number of RF-PFC detected. It was noted that at cell concentrations higher than 3×10^6/slide, a corresponding increase in the number of RF-PFC was not seen. Rather, varying degrees of inhibition were found (Fig. 4). When full tissue culture media were used in the agar instead of the usual balanced salt solution (BSS) (21), however, the expected increment of RF-PFC with increased numbers of cells plaqued did occur (Fig. 5). The inhibition seen in BSS alone (Fig. 4) indicates a nutritional deficiency in the system at higher cell concentrations, where the metabolic demand would be maximal. This interpretation is supported by the further observation that addition of increasing quantities of purified polymorphonuclear leukocytes to the slides with a standard dose of lymphocytes

diminished the number of RF-PFC seen in the preparation.

Blood samples collected in heparin can be kept at 4°C overnight and the cells separated and plaqued the next day without loss of numbers of RF-PFC. If the cells are separated by Ficoll-Hypaque and then stored overnight, however, the number of RF-PFC found the next day is markedly reduced, even if the separated cells are kept in full tissue culture media. Cells separated by Ficoll-Hypaque, and frozen in dimethyl sulfoxide in liquid nitrogen have, on thawing, yielded 33% living cells with no loss of RF-PFC/10^6 living lymphocytes.

Clinical observations. Table II shows our findings on RF-PFC in our first series of RA patients, including both those who were seropositive and those who were seronegative for RF in the serum. Cells from blood, synovial fluid, and BM were examined. 32 of 102 preparations of blood lymphocytes from 21 of 67 patients were positive with a range of 1–18 PFC/10^6 lymphocytes. In this first series we noted that RF-PFC were found especially in two circumstances; when the patients had acute exacerbations of polyarticular synovitis, or when they had generalized vasculitis.

19 synovial fluid lymphocyte preparations were examined, of which 4 were positive with a range of 3–14 PFC/10^6 lymphocytes. RF-PFC were found only in effusions from joints that were the sites of acute synovitis. Joints that were the sites of chronic indolent disease were negative, regardless of the duration of the synovitis or the degree of synovial hypertrophy.

Eight BM preparations were examined. Four patients gave positive cells with a range of 2–22 RF-PFC/10^6 nucleated cells. Two of the four positive BM specimens were from patients who did not have RF-PFC in their peripheral bloods.

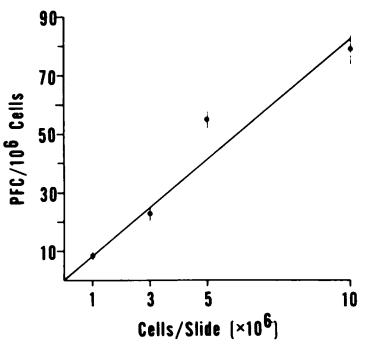

FIGURE 5 RF-PFC at varying cell densities; effect of tissue culture and medium. Effect of supplemental nutrients on numbers of RF-PFC. The indicated numbers of PBL were added to 0.5 ml of agarose in "Autopow" tissue culture medium (Flow Laboratories Inc., Rockville, Md.) supplemented with 10% FBS, 0.033 M $NaHCO_3$, 0.001 M Na pyruvate, 0.002 M L-glutamine, and nonessential amino acids (1/100 of 100X concentrate, Microbiological Associates, Bethesda, Md.).

TABLE II

Occurrences of RF-PFC in Lymphocytes from Three Sources in RA

Source	Total		RF-PFC positive	
	Preparations	Individuals	Preparations	Individuals
Peripheral blood	102	67	32	21
Synovial fluid	19	11	4	3
Bone marrow	8	8	4	4

In Table III, the results in seronegative and seropositive individuals are compared separately. In the seropositive individuals, 40 of 121 peripheral blood, synovial fluid, or BM preparations exhibited one or more RF-PFC/10^6 lymphocytes. No preparations from 25 seronegative patients or from 24 normal persons were positive.

On a number of occasions we examined the blood simultaneously with the BM or joint fluid cells for RF-PFC. As can be seen in Table IV, in all instances the BM had many more RF-PFC/10^6 lymphocytes than did the simultaneous peripheral blood. The synovial fluid lymphocytes sometimes had more RF-PFC. We have taken this to mean that the BM is a source of RF-PFC. The synovial tissues may also be a source, although the inflammation in the joints may conceivably lead to local sinkhole effects which concentrate RF-PFC in the area.

The relation of RF-PFC to severity of disease was investigated in two further series of patients with seropositive RA. The one (second series in Tables V and VI) was a group with mild-to-moderate disease, most of whom were on a drug trial with aspirin, indomethacin, and Naprosyn, and none of whom were on corticosteroids. The other (third series) was a group of patients preselected over a 5-mo period for RF-PFC determinations because of a clinical impression of there being such active, aggressive, or exacerbating disease in them that there was high likelihood that their peripheral blood leukocytes would be positive. The tests on

TABLE III

RF-PFC in Cell Preparations from Individuals Seropositive or Seronegative for RF by the LFT

		RF-PFC	
		Positive	Negative
LFT	Positive	40	81
	Negative	0	49

TABLE IV

RF-PFC in Bone Marrows, Synovial Fluids, and Simultaneously Obtained PBL

Patient	PFC/10^6 lymphocytes		
	Bone marrow	Peripheral blood*	Synovial fluid*
P. D.	640	8	
E. S.	380‡	80	86
V. R.	280	1	
H. R.	220‡	2	
J. R.	53	9	
K. B.	72	3.8	
H. S.	30‡	4	
R. P.		11	51
G. R.		2	16
M. P.		0	15
V. M.		3	5

* Lymphocytes separated by Ficoll-Hypaque gradient.
‡ Numbers of RF-PFC in these patients is based on an assumed value of 10 lymphocytes/100 nucleated BM cells. The differential counts on the BM of the remaining patients revealed 12.5, 10, 8, and 13% lymphocytes, respectively.

the bloods of these latter series were compared with those found among the patients with seropositive RA from our initial series (Table II).

The seropositive RA patients in our initial survey exhibited RF-PFC in their peripheral bloods in 22/67 (33%) of patients (Table V). Most of these had fewer than 5 RF-PFC/10^6 lymphocytes. The group of patients selected to have mild-to-moderate disease showed almost identical findings. The group selected because of more active and aggressive disease, however, had RF-PFC in 10/18 (56%) and in the majority of positives there were more than 5 RF-PFC/10^6 lymphocytes. When patients with clinically diagnosable generalized vasculitis were selected from the three groups and scored as a separate series, it was noted (last column) that all were positive for RF-PFC.

TABLE V

RF-PFC in Three Series of Seropositive RA Patients

PFC/10^6 cells	First series: unselected for severity	Second series: mild or moderate disease	Third series: severe or exacerbating disease	Patients with vasculitis*
15	2	0	6	3
5–15	7	1	1	3
1–5	13	4	3	6
<1	45	11	8	0
Totals	67	16	18	12

* The patients in this column were all those with vasculitis occurring in the three series.

Further evaluation of these three series was carried out in an attempt to better define the clinical parameters that might correlate with presence of RF-PFC. In the second series, which was being followed closely in our Clinical Research Center, the commonly used indicators of disease activity such as number of painful joints, number of hot or swollen joints, hemoglobin and serum albumin levels, and hours of morning stiffness could be evaluated. None of these individually gave positive correlations with RF-PFC. This sort of detailed evaluation could not be made in the first or third series, since these latter groups were not being followed clinically by us, but by cooperating private physicians.

The charts of all three series, however, were evaluated retrospectively for other laboratory data[3] collected simultaneously with the RF-PFC determinations, for ages of patients, and for drug therapy. The results are shown in Table VI. The most striking correlation with RF-PFC in the blood was a markedly elevated erythrocyte sedimentation rate. Very high titers of serum RF and treatment with corticosteroids also correlated with RF-PFC in the summated series, but not reproducibly so in the individual series. There was a tendency for RF-PFC to be found in older patients in all three groups, but at no point did this tendency reach statistical significance.

Serial studies of RF-PFC in the peripheral blood have been carried out in a number of patients. In almost all of these the RF-PFC were assessed for the first time during an exacerbation of disease. Repeatedly we have noted disappearance of RF-PFC as the disease activity subsided. In rare instances we have had opportunity to observe a negative test for RF-PFC during quiescence become positive during an exacerbation.

RF-PFC have not always been identified in the peripheral blood in exacerbating joint disease. On the other hand, they have uniformly been identified in patients with necrotizing vasculitis (Table V). In patients with necrotizing vasculitis, active joint disease is often clinically insignificant. A 71-yr-old patient with "burned out" joint disease is of special interest (Fig. 6). Although this patient had had no specifically active joint disease for several years, she continued to have a high sedimentation rate, nail fold thrombi, diffuse musculoskeletal pain and profound weakness, RF-PFC in her peripheral blood and BM, and a markedly increased catabolism of her IgG.[4] When steroids, which she had

[3] Only 33 of the 67 patients in the first series provided usable information, because the laboratory data sought were not obtained simultaneously with the RF-PFC.

[4] Krick, E. H., N. A. Catalano, R. M. Nakamura, D. H. DeHeer, and J. H. Vaughan. Metabolism of autologous and homologous IgG in rheumatoid arthritis. Manuscript in preparation.

TABLE VI

Clinical Characteristics of Patients Positive and Negative for RF-PFC Detectable in PBL

	Series 1*		Series 2		Series 3		Totals: series 1–3		
	Pos. (20)	Neg. (13)	Pos. (5)	Neg. (11)	Pos. (10)	Neg. (8)	Pos. (35)	Neg. (32)	
	%		%		%		%		*P value*§
Latex > 1,280	62	25	60	27	50	67‡	57	31	0.06
> 640	76	63	80	45	90	83‡	80	60	NS
ESR > 60	33	13	20	0	80	33‡	43	13	<0.02
> 40	71	56	20	27	100	50‡	71	41	<0.05
Age, *yr* > 50	67	56	60	54	70	63	66	56	NS
Steroid Rx	65	55	0	0	80	38	60	31	<0.05

Abbreviation: ESR, erythrocyte sedimentation rate.

Numbers in parentheses represent the number of patients studied.

* On review of the records of 67 patients in this original survey, only 33 had had determinations of RF-PFC, latex, and ESR on the same day, so only the data from the 33 patients are displayed here.

‡ These figures are derived from only six of the eight patients who were RF-PFC negative in this series, since two patients did not have RF-PFC, latex, and ESR done on the same day.

§ *P* values for Pos.-Neg. differences in the totalled series are derived from fourfold contingency tables with Yates' correction (35).

received for many years, were briefly decreased during the early observation period in our Clinical Research Center, she developed diffuse erythema marginata, especially over the back. Serial BM and peripheral blood evaluations were carried out (Fig. 6). Three features are evident: (*a*) the BM regularly exhibited more RF-PFC/10^6 lymphocytes than did the peripheral blood; (*b*) two peaks of RF-PFC occurred in the peripheral blood which did not match changes in BM numbers; and (*c*) there was a lessening and disappearance of RF-PFC in both tissues during treatment with the immunosuppressive agents cyclophosphamide and chlorambucil.

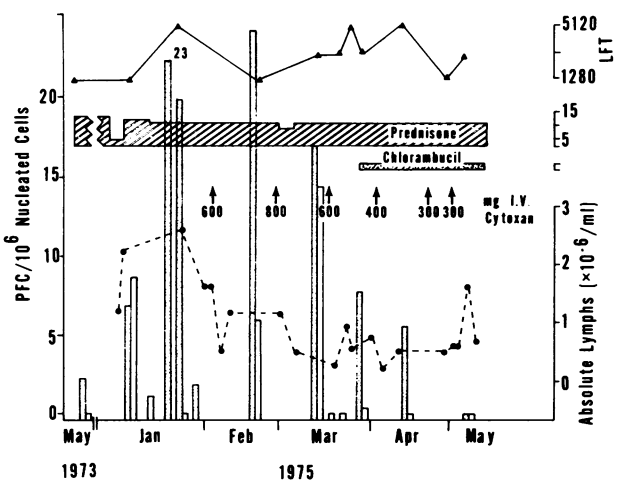

FIGURE 6 Serial observations on a patient with rheumatoid vasculitis. The numbers of RF-PFC/10^6 nucleated BM cells are illustrated in the stippled bars. RF-PFC in PBL are indicated in the open bars. LFT is shown in the solid line. Absolute lymphocyte count is shown in the dashed line. The patient had been under continuous prednisone therapy for 10 yr. The changes accompanying institution of immunosuppressive therapy with cytoxan and chlorambucil can be seen.

Types of extra-articular rheumatoid disease other than necrotizing vasulitis may also be correlated with RF-PFC in the peripheral blood. We have recently observed a patient with "burned out" arthritis, but continued active formation of subcutaneous nodules, Felty's syndrome, a severe bilateral sensory polyneuropathy, a very high sedimentation rate, and a markedly increased catabolic decay of her own IgG.[4] Numerous RF-PFC persistently have been present in her blood.

DISCUSSION

In the RF-PFC assay reported herein, direct hemolysis of sensitized SRC was produced in agar. This is assumed to be due to cells producing complement-fixing IgM-RF (8, 22). Reduced and alkylated IgG hemolysin was used as the SRC sensitizer, since reduced and alkylated hemolysin has no ability by itself to fix complement to the SRC, but remains reactive with RF (9, 23). The plaque assay system is specific for RF. It is inhibited by as little as 2–10 μg of human or rabbit IgG in the agar, less so by bovine IgG, and not at all by 10% FBS.

It was of some interest that the order of the abilities of the various species of IgG to inhibit RF-PFC was different from that by which they inhibited the RF in the blood serums (SCAT). Human IgG was most efficient in the RF-PFC, while rabbit IgG was most efficient in the SCAT. This difference brings up the possi-

bility that the RF that we have traditionally studied in the peripheral blood serum is not properly representative of RF as it is made, but rather it is the residual of the original RF after complexing with interstitial and circulating IgG, precipitation, phagocytosis, or other fate in the tissues. This is obviously an important area for further study.

We have previously speculated that polyclonal IgM-RF may be composed of a spectrum of monoclonal RF molecules, the majority of which are noncomplement fixing, a minority being complement fixing (9). Since noncomplement-fixing IgM-RF would not be detected in our direct hemolytic plaque assay system, we have used a rabbit anti-IgM serum in an attempt to bring out such postulated nonhemolytic RF in an indirect assay. This attempt has been unsuccessful. We used for this attempt, however, an IgG anti-IgM, and the IgG of the antiserum was in sufficient concentration to cause by itself some inhibition of the system such as that illustrated in Fig. 1. An IgM anti-IgM would avoid the problem, and we plan to investigate this question again with such an antiserum. An IgM anti-IgG will also be developed in an attempt to detect IgG RF-PFC.

Demonstration of RF-PFC was optimal when the cells were kept in full tissue culture media for the maximal possible time. The need for continued active protein synthesis and an intact microtubular or membrane structure for optimal RF-PFC production have been indicated by the inhibition studies with cycloheximide and vinblastine.

The clinical findings in these studies are: (*a*) RF-PFC may be found in the peripheral bloods of patients in acute exacerbations, or with persistent aggressive disease such as necrotizing vasculitis; (*b*) the BM is a good source of RF-PFC, sometimes exhibiting them when the peripheral blood is free of them; and (*c*) the synovial fluid may also exhibit RF-PFC in acute flares of synovitis, at which time it too may have more PFC/10^6 lymphocytes than the peripheral blood.

The BM has long been known to be a source of lymphocytes capable of repopulating thymic, splenic, and nodal organs (24). It is also an organ capable of considerable antibody (25) and gamma globulin (26) production. We have previously reported in systemic lupus erythematosus anti-DNA PFC in high numbers in the BM of patients with acute exacerbations (27) of that disease.

The higher numbers of RF-PFC in joint fluids than in simultaneously obtained peripheral bloods may mean local RF-PFC production in the joints; but it also may mean local entrapment of RF-PFC from the circulating blood. In a study of autoimmune thyroiditis in the rabbit, Clinton and Weigle (28) have shown accumulation of PFC making autoantibody to thyroglobin in the inflamed gland. They suggested that locally produced thyroglobulin could act as an affinity absorbent for anti-thyroglobulin-producing PFC. Later studies by Clagett et al. (29) emphasized that such local accumulation of PFC could also provide a means of delivering large amounts of autoantibody into the immediate vicinity of the gland with resultant local immune precipitation with thyroglobulin. Whether the synovial tissues, which are known to be capable of IgG production (8), act similarly as an affinity absorbent for RF-PFC and precipitant for their RF is a possibility that needs further study.

The intermittent appearance of RF-PFC in the peripheral blood in RA has a parallel in experimental animals undergoing active immunization. PFC to various immunizing antigens appear in the peripheral bloods of animals only for the brief periods during which there is active proliferation of new antibody-producing cells by the lymphoid tissues (30–34). This was noted in mice at about 5 and 13 days after primary immunization with aggregated human IgG by Romball and Weigle (34). Serum antibody titers did not correspond with the numbers of PFC in the peripheral blood, but rather lagged after the PFC. Sometimes the serum antibody level continued to rise after the peripheral blood PFC disappeared, indicating continuing antibody production by cells in the fixed lymphoid tissues.

We have observed a number of patients with particularly active and aggressive disease in whom the peripheral blood was persistently positive for RF-PFC, but with change from positive to negative during therapy. Except for one case, all showed improvement of clinical symptoms on therapy. We have also seen patients spontaneously lose their peripheral blood RF-PFC while spontaneously improving clinically. Fall in the RF-PFC often occurred either with no change in the RF titer of the serum, or with a diminution that lagged considerably after the fall in RF-PFC. Thus, we confirm the well-recognized failure of the titer of serum RF to correlate well with changes in disease activity. RF-PFC in the peripheral blood, however, shows a closer correlation with disease activity.

We note that the intermittency with which RF-PFC are seen in the blood of RA patients may reflect an intermittency in an immunizing process in their disease. We note also that the qualitative differences of the RF detected in the RF-PFC assay from the RF detected in the SCAT may indicate selective removal of portions of RF from the body fluids, and this asks for attention to the possible role of this portion of the RF in the pathogenesis of RA.

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REFERENCES

1. Mellors, R. C., R. Heimer, J. Corcos, and L. Korngold. 1959. Cellular origin of rheumatoid factor. *J. Exp. Med.* **110**: 875–886 and Plates 92–96.
2. Mellors, R. C., A. Nowoslawski, L. Korngold, and B. L. Sengson. 1961. Rheumatoid factor and the pathogenesis of rheumatoid arthritis. *J. Exp. Med.* **113**: 475–484 and Plates 49–54.
3. McCormick, J. N. 1963. An immunofluorescence study of rheumatoid factor. *Ann. Rheum. Dis.* **22**: 1–10.
4. Bonomo, L., A. Tursi, and U. Gillardi. 1968. Distribution of the anti-gamma globulin factors in the synovial membrane and other tissues in various diseases. *Ann. Rheum. Dis.* **27**: 122–129.
5. Milde, E-J., and O. Tönder. 1968. Demonstration of rheumatoid factor in tissue by mixed agglutination with tissue sections. *Arthritis Rheum.* **11**: 537–545.
6. Bach, J-F., F. Delrieu, and F. Delbarre. 1970. The rheumatoid rosette. A diagnostic test unifying seropositive and seronegative rheumatoid arthritis. *Am. J. Med.* **49**: 213–222.
7. Durance, R. A., A. Micheli, and F. H. Fallet. 1974. Rosette formation in rheumatoid and non-rheumatoid states. *Ann. Rheum. Dis.* **33**: 216–220 .
8. Smiley, J. D., C. Sachs, M. Ziff. 1968. In vitro synthesis of immunoglobulin by rheumatoid synovial membrane. *J. Clin. Invest.* **47**: 624–632.
9. Tanimoto, K., N. R. Cooper, J. S. Johnson, and J. H. Vaughan. 1975. Complement fixation by rheumatoid factor. *J. Clin. Invest.* **55**: 437–445.
10. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody producing cells. *In* Cell Bound Antibodies. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia. 109.
11. A Committee of the American Rheumatism Association. 1958. Revision of diagnostic criteria of rheumatoid arthritis. *Arthritis Rheum.* **2**: 16–20.
12. Hess, E. V. 1974. A standard database for rheumatic diseases. *Arthritis Rheum.* **17**: 327–336.
13. Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97): 77–89.
14. Cassen, B., J. Hitt, and E. F. Hays. 1958. The efficient separation of lymphocytes from human blood. *J. Lab. Clin. Med.* **52**: 778–783.
15. Levine, S. 1956. Magnetic techniques for in vitro isolation of leukocytes. *Science (Wash. D. C.).* **123**: 185–186.
16. Heller, G., A. S. Jacobson, and M. H. Kolodny. 1949. A modification of the hemagglutination test for rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med.* **72**: 316–323.
17. Butler, V. P., Jr., and J. H. Vaughan. 1965. The reaction of rheumatoid factor with animal gamma-globulins: Quantitative considerations. *Immunology.* **8**: 144–159.
18. Butler, V. P., Jr., and J. H. Vaughan. 1964. Hemagglutination by rheumatoid factor of cells coated with animal gamma globulins. *Proc. Soc. Exp. Biol. Med.* **116**: 585–593.
19. Waller, M. V., and J. H. Vaughan. 1956. Use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med.* **92**: 198–200.
20. Bensch, K. G., and S. E. Malawista. 1969. Microtubular crystals in mammalian cells. *J. Cell. Biol.* **40**: 95–107.
21. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**: 423–442.
22. Schmid, F. R., and M. J. Rocha. 1968. Complement fixation by IgM antibody (rheumatoid factor): Dependence on antigen concentration. *J. Lab. Clin. Med.* **72**: 1014–1015. (Abstr.)
23. Zvaifler, N. J., and P. Schur. 1968. Reactions of aggregated mercaptoethanol treated gamma globulin with rheumatoid factor—Precipitation and complement fixation studies. *Arthritis Rheum.* **11**: 523–536.
24. Micklem, H. S., C. E. Ford, E. P. Evans, and J. Gray. 1966. Interrelations of myeloid and lymphoid cells: Studies with chromosome-marked cells transfused into lethally irradiated mice. *Proc. R. Soc. Lond B. Biol. Sci.* **165**: 78–102.
25. Benner, R., F. Meima, G. M. van der Meulen, and W. B. van Muiswinkel. 1974. Antibody formation in mouse bone marrow. I. Evidence for the development of plaque-forming cells *in situ*. *Immunology.* **26**: 247–255.
26. McMillan, R., R. L. Longmire, R. Yelenosky, J. E. Lang, V. Heath, and C. G. Craddock. 1972. Immunoglobulin synthesis by human lymphoid tissues: Normal bone marrow as a major site of IgG production. *J. Immunol.* **109**: 1386–1394.
27. Bell, D. A., C. Clark, S. E. Blomgren, and J. H. Vaughan. 1973. Anti DNA antibody production by lymphoid cells of NZB/W mice and human systemic lupus erythematosus (SLE). *Clin. Immunol. Immunopathol.* **1**: 293–303.
28. Clinton, B. A., and W. O. Weigle. 1972. Cellular events during the induction of experimental thyroiditis in the rabbit. *J. Exp. Med.* **136**: 1605–1615.
29. Clagett, J. A., C. B. Wilson, and W. O. Weigle. 1974. Interstitial immune complex thyroiditis in mice: The role of autoantibody to thyroglobulin. *J. Exp. Med.* **140**: 1439–1456.
30. Friedman, H. 1964. Distribution of antibody plaque forming cells in various tissues of several strains of mice injected with sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* **117**: 526–530.
31. Hiramoto, R. N., N. M. Hamlin, and H. L. Harris. 1968. The detection of antibody-containing cells in the blood stream during the IgM cellular antibody response. *J. Immunol.* **100**: 637–640.
32. Roseman, J. M., L. D. Leserman, F. W. Fitch, and D. A. Rowley. 1969. Do antibody-forming cells circulate in the blood? *J. Immunol.* **102**: 1002–1007.
33. Luzzati, A. L., I. Lefkovits, and B. Pernis. 1973. Antibody response by rabbit peripheral blood lymphocytes in microcultures. *Eur. J. Immunol.* **3**: 632–635.
34. Romball, C. G., and W. O. Weigle. 1973. A cyclical appearance of antibody-producing cells after a single injection of serum protein antigen. *J. Exp. Med.* **138**: 1426–1442.
35. Mainland, D. 1964. Comparison of samples of frequency data. *In* Elementary Medical Statistics. W. B. Saunders Co., Philadelphia. 2nd edition. **XII**: 224.