Effect of drug therapy on circulating and synovial fluid Ig-secreting cells in rheumatoid arthritis

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SUMMARY A common immunological abnormality in rheumatoid arthritis (RA) is an increased spontaneous polyclonal B cell activation. In order to study the influence of drug therapy in RA on the functional activity of B cells we enumerated spontaneous plaque-forming cells (PFC) in peripheral blood lymphocytes (PBL) and synovial fluid lymphocytes (SFL) by a reverse haemolytic plaque assay. Spontaneous IgG-, IgM-, and IgA-PFC in PBL of 26 patients with classical erosive RA receiving either gold salts or D-penicillamine were similar to those observed in 20 healthy controls. In contrast, significantly higher numbers of IgG- and IgA-PFC, but not IgM-PFC, were found in PBL of nine patients with classical erosive RA receiving non-steroidal anti-inflammatory drugs (NSAID) alone. Furthermore, spontaneous PFC in SFL from 16 consecutive patients with RA receiving second-line drugs, as well as 17 patients with other forms of arthritis (non-RA) were generally low and significantly less than those observed in 20 RA patients on NSAID alone. Moreover, a wide individual variation in PFC, especially in relation to the IgG class, was recorded in the synovial lymphocytes. These studies imply that treatment with second-line drugs is associated with normalisation of B cell activity in RA patients, and that the effect can be detected at the cellular level both in blood and synovial fluid.

Key words: antibody formation, gold thiomalate, D-penicillamine, non-steroidal anti-inflammatory drugs.

In rheumatoid arthritis there is ample evidence of increased activity in the antibody forming cell system. This was exemplified by increased spontaneous B cell proliferation,^{1 2} increased background Ig-secreting cells,^{3 4} and polyclonal Ig secretion including autoantibodies.⁵⁻⁷ Although the cause of B cell hyper-reactivity is still unknown, viral and bacterial products with polyclonal B cell activating (PBA) properties have been implicated.⁸ A very high proportion of activated B cells in the rheumatoid synovium secrete rheumatoid factor (RF), which has been postulated to play an important role in the pathogenesis of rheumatoid injury.^{9 10}

Treatment with gold salts and penicillamine has long been shown to improve both the clinical and laboratory findings in patients with RA.¹¹⁻¹³ A tendency for normalisation in the serum level of

immunoglobulins, as well as other disease characteristics, such as RF and immune complexes, has been found during effective treatment with gold salts and penicillamine.¹⁴⁻¹⁸

By the protein A plaque assay, which detects Ig-secreting cells, we have previously demonstrated the presence of markedly increased numbers of spontaneous Ig-secreting cells in blood and synovial fluids of patients with active seropositive RA who were not receiving gold, penicillamine, corticosteroid, or cytostatic drugs.³ The present paper is an extension of our previous observations with the aim of studying the effect of NSAID, compared with gold salts and D-penicillamine, on circulating and synovial fluid Ig-secreting cells in RA patients.

Materials and methods

PATIENTS AND CONTROLS

Eighty-eight patients and 20 healthy nursing and medical staff from the Division of Rheumatology,

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Department of Internal Medicine, Danderyd Hospital, Stockholm, were studied.

The first group included 35 patients selected on the basis of their clinical findings and therapy, in whom numbers of circulating PFC were analysed and compared with 20 healthy controls. All patients fulfilled the American Rheumatism Association criteria for classical RA¹⁹ and had typical erosive radiographic abnormalities. Thirty-two patients had positive tests for RF on at least two occasions during their illness, whereas the other three had only one positive test. Nine patients were receiving NSAID, 13 were on gold sodium thiomalate (Myocrisin) and another 13 on D-penicillamine (Dista Products). In order to ensure compatibility of the patients and in view of the reported differences between classical erosive and non-erosive RA²⁰⁻²² only patients with classical erosive seropositive RA were included in this group. As expected, several had negative tests for RF at the time of this study (Table 1) following a period of drug therapy.^{14 15} Further clinical and laboratory characteristics are presented in Table 1. Control subjects included 10 females and 10 males with an average age of 39.1 years (SD 3.4, range 20-63).

The second group included 53 consecutive patients with various forms of arthritis, in whom numbers of spontaneous PFC were analysed simultaneously in blood and synovial fluid. These included: (1) 20 patients with RA (12 classical and eight definite) who were receiving NSAID alone; (2) 16 patients with RA (seven classical and nine

definite) who were receiving second-line drugs; and (3) 17 patients with non-RA (14 on NSAID and three on less than 7.5 mg/day prednisolone). The clinical diagnosis, treatment, and other features of the patients included in this group are summarised in Table 2.

SYNOVIAL FLUID PREPARATION

All samples were collected aseptically in heparinised tubes ready for transport, which usually took about one hour. The fluid was immediately mixed with 10 mg/l hyaluronidase (Sigma Chemical Co., St Louis, Mo, USA) at 37°C for 45 min.

CELL ISOLATION

Mononuclear cells were isolated from heparinised venous blood or synovial fluid by centrifugation on a Ficoll-Isopaque (Nyegaard, Oslo) gradient.²³ The cells recovered at the interphase were removed, washed four times with Hanks's balanced salt solution (HBSS), and resuspended at $0.8-1.2 \times 10^9$ cells/l viable cells, as judged by the trypan blue exclusion test. Cell suspensions were always kept on ice until used.

ASSAY FOR PLAQUE-FORMING CELLS

Ig-secreting cells in lymphocyte suspensions were enumerated by a haemolysis-in-gel technique,²⁴ as modified by Gronowicz *et al.*,²⁵ with protein A-coupled sheep erythrocytes as target cells. Plaques were developed with a monovalent IgG fraction of rabbit antihuman Ig specific for γ , μ , or α -chains

Table 1 Clinical and laboratory characteristics of 35 patients with classical erosive rheumatoid arthritis in whom circulating PFC were determined

Parameter	Patient group		
	Gold	D-Penicillamine	NSAID
Number of patients	13	13	9
Female:male	4:9	9:4	5:4
Age (years)	54.3 $\pm$ 3.3* (40-71) [†]	60.9 $\pm$ 2.4 (43-70)	67.0 $\pm$ 2.4 (44-76)
Disease duration (years)	5.1 $\pm$ 0.9 (1-13)	14.5 $\pm$ 2.6 (3-34)	7.0 $\pm$ 1.8 (2-14)
Drug: dose	94 $\pm$ 20 (10-200) mg/month	548 $\pm$ 66 (250-1000) mg/day	NE [‡]
duration (months)	18.8 $\pm$ 7.1 (2-94)	67.5 $\pm$ 7.3 (9-84)	NE
Extra-articular manifestations	3	1	2
RF $\geq$ 1/40§	11	6	7
ANA $\geq$ 1/25	2	3	2
ESR $\geq$ 28 mm/h	6	1	9
Serum IgG (g/l)	11.49 $\pm$ 0.94	11.91 $\pm$ 0.73	14.30 $\pm$ 1.13
Serum IgM (g/l)	1.12 $\pm$ 0.19	1.12 $\pm$ 0.17	1.14 $\pm$ 0.27
Serum IgA (g/l)	1.87 $\pm$ 0.23	1.89 $\pm$ 0.20	1.98 $\pm$ 0.32
P-haptoglobin (g/l)	2.00 $\pm$ 0.30	1.61 $\pm$ 0.11	2.08 $\pm$ 0.28
Orosomucoid (g/l)	0.68 $\pm$ 0.08	0.50 $\pm$ 0.02	0.72 $\pm$ 0.08

*Mean $\pm$ SEM. [†]Range. [‡]Not evaluated. §At the time of sampling.

ANA=antinuclear antibodies.

Normal values: serum IgG, 6.2-15 g/l; serum IgM, 0.40-2.75 g/l; serum IgA, 0.8-4.0 g/l; P-haptoglobin, 0.20-1.40 g/l; orosomucoid, 0.25-0.65 g/l.

Table 2 Major characteristics of 53 consecutive patients with rheumatoid and non-rheumatoid arthritides, in whom circulating as well as synovial fluid PFC were analysed simultaneously

Diagnosis	n	Age (yr)	Sex	RF ≥1/40	ESR ≥28	Treatment
RA	20	59.7±3.2*	12F, 8M	11	18	NSAID
RA	7	61.9±4.2	5F, 2M	2	5	Penicillamine
RA	2	61.5±3.5	1F, 1M	1	1	Gold
RA	3	66.0±4.5	3F	2	3	Prednisolone
RA	4	43.0±3.3	4F	1	2	Chloroquine
Gout	4	49.5±9.1	3F, 1M	0	1	NSAID
Pelvospondylitis	1	37	1M	0	1	Prednisolone
Juvenile recurrent polyarthritis	3	20.0±2.7	2F, 1M	0	3	2 Prednisolone 1 NSAID
Reactive arthritis	3	30.0±6.0	1F, 2M	0	0	NSAID
Enteroarthritis	1	62	1F	0	0	NSAID
Seronegative oligoarthritis	5	38.6±9.5	4F, 1M	0	2	NSAID

*Mean±SEM.

ESR=erythrocyte sedimentation rate.

(DAKO Immunoglobulins Ltd, Copenhagen, Denmark) diluted 1:30. The detailed procedure has been described previously.³

STATISTICAL ANALYSIS

Student's *t* test was used for comparing means between groups. Since spontaneous PFC in synovial fluids were not normally distributed, mean values of the natural logarithms of the PFC numbers were used. Probability values (*p* values) were determined in two-tailed tests. When two independent groups had similar variance a pooled variance estimate of *t* was used, otherwise a separate variance estimate of *t* with an estimated number of degrees of freedom was used to test whether or not the means of the groups differed. Correlation was analysed by Pearson's product-moment correlation coefficients.

Results

EFFECT OF DRUG THERAPY ON SPONTANEOUS PFC IN RA BLOOD: COMPARISON WITH NORMAL CONTROLS

Spontaneous IgG-, IgM-, and IgA-PFC were enumerated in PBL isolated from 20 healthy controls and 35 patients with classical erosive RA who were receiving either gold salts, D-penicillamine, or NSAID alone. The major characteristics of the patient groups are given in Table 1. The average numbers (mean ± SEM) and the pattern of distribution of different Ig classes of the patient and control groups are presented in Table 3 and Fig. 1. It can be seen that patients receiving either gold salts or D-penicillamine had numbers and distribution of spontaneous PFC similar to those found in healthy controls (*p* > 0.05 for all tested Ig classes). Con-

Table 3 Effect of drug therapy on circulating PFC (mean±SEM) in rheumatoid arthritis. Comparison with healthy controls. The *p* values between different groups are given for each Ig class

Patient	PFC (mean±SEM)/10 ⁶ viable cells				
	n	IgG	IgM	IgA	Total
1. RA+NSAID	9	1018±210	296±45	1961±340	3275±452
2. RA+gold	13	294±76	148±51	624±128	1066±206
3. RA+penicillamine	13	325±115	241±62	1102±218	1668±302
4. Normal controls	20	218±29	166±49	990±131	1374±167
<i>p</i> value					
1 v 2		<0.01	NS*	<0.01	<0.001
1 v 3		<0.01	NS	<0.05	<0.01
2 v 3		NS	NS	NS	NS
4 v 1		<0.01	NS	<0.05	<0.01
4 v 2		NS	NS	NS	NS
4 v 3		NS	NS	NS	NS

*Not significant.

Fig. 1 Distribution of circulating IgG-, IgM-, and IgA-PFC in 20 healthy controls and 35 RA patients receiving either gold salts, D-penicillamine, or NSAID alone. The means and the standard errors of the means are indicated for each group. The *p* values between different groups are given in Table 3.

versely, patients receiving NSAID alone had significantly more PFC than normal controls ($p < 0.01$) and patients treated with either gold ($p < 0.001$) or D-penicillamine ($p < 0.01$). These differences were significant for IgG- and IgA-PFC but did not reach a significant level for IgM-PFC. There was no significant difference in PFC numbers between groups on gold salts and D-penicillamine. Thus treatment with gold salts and D-penicillamine, but not NSAID alone, is associated with normalisation of the numbers of Ig-secreting cells in RA blood.

Analysis of the data (Pearson's product-moment correlation coefficients) showed that there was a significant correlation between the total numbers of spontaneous PFC and ESR ($r = 0.40$, $p = 0.02$). The correlation was significant for IgG-PFC ($r = 0.46$, $p = 0.006$), did not reach a significant level for IgA-PFC ($r = 0.30$, $p = 0.08$), and was not significant for IgM-PFC ($r = 0.14$, $p = 0.42$). It should be pointed out that the mean levels ($\pm$ SEM) of ESR were significantly higher among patients receiving NSAID alone (57.7 ± 7.6) than patients treated with either gold (32.8 ± 7.6 , $p = 0.04$) or penicillamine (19.8 ± 3.1 , $p = 0.001$). In the present study no significant correlation was found between the numbers of spontaneous PFC and age, sex, disease duration, extra-articular manifestations, serum levels of Ig, RF titres, P-haptoglobin, or orosomucoid.

EFFECT OF DRUG THERAPY ON SPONTANEOUS PFC IN RA SYNOVIAL FLUID: COMPARISON WITH NON-RA

In earlier studies SFL from untreated patients with RA were found to contain high numbers of spontaneous PFC.³ In the present study SFL from three groups of patients have been compared: (1) RA patients receiving NSAID alone; (2) RA patients treated with second-line drugs (gold, D-penicillamine, corticosteroid, or chloroquine); and (3) non-RA patients. Further details are given in Table 2. As shown in Table 4, a significantly higher number of spontaneous PFC was found in RA patients receiving NSAID alone compared with those treated with second-line drugs ($p < 0.01$). Furthermore, spontaneous PFC in the non-RA synovial fluids were generally low and significantly lower than those obtained from RA patients receiving NSAID alone ($p < 0.01$). There was no significant difference in PFC numbers between non-RA patients and RA patients on second-line drugs. IgG was the predominant Ig-class in all synovial fluids tested (Table 4). Fig. 2 shows that spontaneous PFC in synovial fluids from all patients with non-RA and RA patients treated with second-line drugs lie within a range ≤ 100 – $3817/10^6$ SFL. A similar range of PFC was also observed in 12 of the 20 RA patients receiving NSAID alone. In contrast, eight RA patients on NSAID alone had 4950–50 625 PFC/ 10^6 SFL.

Table 4 Effect of drug therapy on synovial fluid PFC (mean±SEM) in rheumatoid arthritis. Comparison with non-rheumatoid arthritis. The *p* values between different groups are given for each Ig class

Patient	PFC (mean±SEM)/10 ⁶ viable cells				
	<i>n</i>	IgG	IgM	IgA	Total
1. RA+NSAID	20	5597±2076	2037±755	958±288	8592±2977
2. RA+second-line drugs*	16	656±224	194±75	188±68	1038±325
3. Non-RA†	17	545±178	59±26	196±120	800±275
<i>p</i> value					
1 v 2		<0.05	<0.05	<0.05	<0.01
1 v 3		<0.01	<0.001	<0.01	<0.01
2 v 3		NS‡	NS	NS	NS

*Two gold; seven D-penicillamine; three prednisolone; and four choloroquine.

†Fourteen NSAID and three prednisolone.

‡Not significant.

Fig. 2 Spontaneous PFC (IgG+IgM+IgA) measured simultaneously in blood and synovial fluid lymphocytes isolated from 53 consecutive patients with rheumatoid and non-rheumatoid arthritis. Each line represents one patient. The means and the standard errors of the means are indicated for each group. Note: Spontaneous PFC in PBL of one patient from the non-RA group were not determined.

When data obtained from synovial fluids were compared with those obtained simultaneously from blood, comparable numbers or, surprisingly, much fewer PFC were found in SFL than in PBL for all patients with non-RA, for RA patients treated with second-line drugs, and for 12 of the 20 RA patients receiving NSAID alone (Fig. 2). In contrast, eight RA patients on NSAID had two- to 10-fold more PFC in SFL than PBL indicating local production of Ig in the rheumatoid synovium.

Correlation of PFC in synovial fluid to various clinical and laboratory parameters indicated that it was not related to age, sex, disease duration, erosive lesions, RF titres, P-haptoglobin, or orosomucoid. However, a slight but significant correlation be-

tween IgM- and IgG-PFC and the occurrence of extra-articular manifestations was found ($r=0.33$, $p=0.01$ for IgM; $r=0.30$, $p=0.03$ for IgG; $r=0.18$, $p=0.21$ for IgA). Although synovial fluid PFC/10⁶ cells were not correlated with ESR ($r=0.21$, $p=0.14$), spontaneous PFC in the PBL isolated from the same patients were significantly correlated with the ESR levels ($r=0.42$, $p=0.002$). Furthermore, spontaneous PFC in synovial fluids were positively correlated with serum levels of IgM ($r=0.42$, $p=0.004$) and IgG ($r=0.35$, $p=0.01$) but not IgA ($r=0.19$, $p=0.21$) antibodies.

Discussion

The plaque assay techniques have been widely used in recent years for the study of immunoglobulin secretion in diseases associated with B cell abnormalities such as RA,^{3,4} systemic lupus erythematosus,^{26,27} and multiple sclerosis.²⁸ This method provides information about the functional state of the B cells within a few hours of their collection and, therefore, should be more reliable than determination of cumulative Ig levels in serum and joint fluid which are influenced by the rate of Ig catabolism and excretion.²⁹ Thus we used the protein A plaque assay to evaluate the effect of drug therapy in RA on the capacity of blood and synovial fluid B lymphocytes to secrete Ig spontaneously.

Our data indicated normal numbers and distribution of circulating Ig-secreting cells in RA patients treated with gold salts or D-penicillamine. As expected, significantly higher numbers of spontaneous Ig-secreting cells, particularly those secreting IgG and IgA antibodies, were found in PBL of patients receiving NSAID alone. These results indicated that treatment with gold salts and D-penicillamine, but not NSAID alone, is associated with normalisation of B cell activity in the RA blood. Using a similar technique, Pardo and

Levinson³⁰ have also demonstrated the presence of normal numbers of circulating PFC in RA patients receiving gold therapy. However, the effect of D-penicillamine therapy on active antibody producing cells has not previously been described. Previous reports about the effect of treatment with gold and penicillamine on serum levels of Ig have presented variable results: some investigators found no effect on serum Ig levels,³¹ while others demonstrated a significant reduction in all major Ig classes (G, M, and A).^{17 32} Furthermore, a selective effect on certain Ig classes has also been reported, though this is variable.^{14 16 33}

The functional activity of lymphocytes isolated from synovial fluids is thought to reflect the inflammatory process in the affected joint. Recent studies with immunohistochemical staining techniques have demonstrated a special relationship between different cell populations infiltrating the rheumatoid synovial membranes, which could ultimately lead to the activation of B lymphocytes.^{34 35} Therefore it is not surprising to find a high proportion of synovial fluid B lymphocytes to be activated to form Ig-secreting cells. In the present report this was particularly pronounced in eight RA patients on NSAID, where 0.5–5% of the total synovial cells were actively engaged in antibody secretion. Among these patients two- to 10-fold more PFC were found in SFL than in PBL, indicating local production of Ig in the rheumatoid synovium. Such high numbers of synovial fluid PFC were not found in any of our RA patients receiving second-line drugs or patients with other forms of arthritis such as gout, pelvispondylitis, juvenile recurrent arthritis, reactive arthritis, enteroarthritis, and seronegative oligoarthritis. A high rate of Ig secretion by rheumatoid synovial cells was originally described by Smiley *et al.*³⁶ and later confirmed by other investigators.^{3 4 37} It should be noted that there was a considerable overlap in synovial fluid PFC between the RA patients on NSAID and the other two groups of patients (Fig. 2) and, therefore, these differences are quantitative rather than qualitative. Furthermore, the data do not necessarily link the high synovial fluid PFC to diagnosis but rather to a state of local inflammation.

We have also noticed a wide individual variation in the number of SFL spontaneously secreting Ig (range ≤ 0.01 –5%) (Fig. 2). Similar variations in PFC numbers (range 0.20–2.76%) have been reported in mononuclear cells eluted from 17 rheumatoid synovial tissues.⁴ Though we do not have an immediate explanation for this phenomenon, it is tempting to relate it to the presence of transient severe synovitis with subsequent release of helper factors triggering a higher proportion of B cells to

secrete Ig. This assumption is supported by our recent demonstration of a helper factor with T cell-replacing activity in the synovial fluid of many, but not all, RA patients.³⁸ Subsequent studies have demonstrated that helper activities in synovial fluids were induced by active molecules distinct from materials that preferentially bind to the staphylococcal protein A, such as IgG-immune complexes, and therefore are probably produced by activated T cells in the rheumatoid synovium.³⁹

Though PFC of different Ig classes were found in SFL, more than 60% were IgG-secreting cells. This was true whether the patients had rheumatoid or other forms of arthritis and whether small or high numbers of PFC were recorded. Thus a predominance of IgG-secreting cells seems to be a special characteristic of synovial fluid B lymphocytes. A predominance of IgG antibody in the rheumatoid synovial space has been previously reported at the cellular level,^{3 4} as well as in synovial fluids and supernatants of cultured cells.^{36 37} The majority of IgG-containing plasma cells in the rheumatoid synovial membranes synthesise IgG-RF.⁹

The numbers of circulating PFC, particularly the IgG-PFC, were found to be significantly correlated with the ESR, which is in agreement with other reports.^{33 40} However, we could not find a significant correlation between synovial fluid total PFC/10⁶ and ESR levels. Instead, increased numbers of synovial fluid PFC were correlated significantly with the occurrence of extra-articular manifestations, which might reflect the severity of the disease process in general.

To summarise, our data are consistent with the concept that B cells are spontaneously activated in RA patients. In addition, treatment with gold salts and D-penicillamine was found to be associated with low numbers of circulating as well as synovial fluid Ig-secreting cells. Though we have not studied the mechanism by which gold salts and D-penicillamine cause a fall in the numbers of Ig-secreting cells, our data provide an indirect clue that both drugs act on the cellular component of the immune system, either directly on B cells or indirectly via T cells or macrophages. Indeed, Lipsky,⁴¹ in an in-vitro study, has demonstrated that D-penicillamine in the presence of copper ions had a selective inhibitory action on helper T cells but no direct effect on B lymphocytes.

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