Immune complexes and the pathogenesis of neutropenia in Felty's syndrome

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SUMMARY The effect of the injection of serum from patients with rheumatoid arthritis (RA) and Felty's syndrome (FS) into mice on the number of circulating polymorphonuclear cells (PMN) was studied. The number of circulating PMN dropped to 61% (range 34–98%) of the initial counts after the injection of FS serum. This phenomenon was observed less frequently after injection of RA serum. In contrast, injection of serum from healthy controls always resulted in an immediate increase in the number of circulating PMN. No decrease in PMN counts was found after injection of FS sera pretreated with polyethylene glycol to precipitate immune complexes (IC). Gel filtration of FS sera on Sepharose 4B showed that the effect on the PMN counts in mice did not coincide with the 7S peak but occurred only in fractions containing larger material. Serum fractions from FS patients that contained IC were more active in producing neutropenia than the corresponding fractions from patients with RA. Microscopic and immunohistochemical examination of the organs from mice injected with FS serum showed sequestration of PMN and deposition of human IgG, IgA, and IgM in the vascular bed of the lungs. These results indicate that the interaction between PMN and IC of patients with FS leads to sequestration of PMN in mice and suggests that this interaction in humans may have a role in the pathogenesis of FS.

Since Felty first described the association of rheumatoid arthritis (RA) with splenomegaly and leucopenia (mainly due to neutropenia)¹ several mechanisms have been suggested for the pathogenesis of this syndrome. Although impaired granulopoietic activity has been reported,^{2,3} the products of activation of the humoral immune system may lead to shortened survival and increased sequestration of polymorphonuclear cells (PMN) in a marginated pool.^{4–6} Sequestration or reduced survival of PMN, or both, in patients with Felty's syndrome (FS) have been associated with an increased binding of immunoglobulins to PMN. These PMN bound immunoglobulins have been defined as auto-antibodies^{7–13} but were recently characterised as immune complexes (IC) bound to Fc and C3 receptors on the membrane.^{14–16} Whether this *in vivo* interaction of circulating PMN and IC leads to neutropenia in FS is still uncertain.

The role of IC in relation to circulating PMN has been studied in animals. Intravenous injection of IC formed *in vitro* in animals caused a transient neutropenia concomitantly with sequestration of PMN in the microvascular bed.^{17,18} These observations suggest that the low numbers of circulating PMN in FS are due to binding of IC to PMN. High levels of circulating IC have been shown, however, in the serum from many RA patients without neutropenia. The present study was designed to investigate possible differences between the sera of patients with FS and RA by measuring their effect on the number of circulating leucocytes after injection into mice.

Materials and methods

PATIENTS AND SERA

The serum samples were derived from six patients with definite RA according to the American Rheumatism Association criteria, eight patients with FS (definite RA with spontaneous sustained neutropenia below 2.0×10^9 PMN/l and splenomegaly), and four healthy controls (HC). Blood was

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allowed to clot at room temperature for one hour and serum was stored in 1 ml aliquots at -20°C until use. The latex fixation and Waaler-Rose tests for rheumatoid factor were positive in all patients. Bone marrow aspirate of all FS patients showed a normal cellularity with normal percentages of mature PMN and PMN precursors. Clinical data of the patients with RA and FS are given in Table 1.

FRACTIONATION OF SERA TO ISOLATE IMMUNE COMPLEXES

Sera from six patients (three with RA and three with FS; Table 1) were fractionated by gel filtration as follows: samples of serum (5 ml) were centrifuged at 2000 g for 10 min and applied to a 2.5×90 cm column of Sepharose 4B (Pharmacia Fine Chemicals, Woerden, The Netherlands). The column was eluted at 6–7 ml/h with veronal buffered saline containing 0.15 M NaCl and 2 mM ethylenediaminetetra-acetic acid, and 5 ml fractions were collected. The protein elution profile was assessed by measuring the optical density at 280 nm of the fractions, which were then combined in two pools, each of which was concentrated by ultrafiltration to the original volume of the fractionated sample and stored until use at -20°C . The IgG and IgM contents of the pools were measured with a sandwich enzyme linked immunosorbent assay for human immunoglobulins.¹⁹ In short, microwells were coated with rabbit IgG antihuman IgG or IgM and incubated with samples of the pools. Bound human immunoglobulins were detected by adding peroxidase conjugated rabbit IgG antihuman IgG or IgM (Dakopatts, Copenhagen, Denmark) followed by 100 $\mu\text{g/ml}$ *o*-phenylenediamine (Sigma Chemicals, St Louis, Mo) in 0.05 M phosphate (pH 5.6) (Dakopatts) containing 0.02% H_2O_2 .

IMMUNE COMPLEXES

IC were precipitated by adding 3% (w/v) polyethylene glycol (PEG 6000, BDH Chemicals Ltd, Poole, England) to serum from patients with FS. PEG was dissolved in 0.1 M boric acid, 0.025 M disodium tetraborate, and 0.075 M NaCl (pH 8). After mixing of the contents the tubes were left on ice for 60 min and then centrifuged at 2400 g at 4°C for 20 min.

The IC content of all patient sera was measured by the C1q binding assay (C1qBA),²⁰ the amount of IC was expressed as micromoles of a standard of aggregated IgG.²¹ Human IgG was isolated from heat inactivated donor serum by ammonium sulphate precipitation and anion exchange chromatography on diethylaminoethyl-cellulose. Aggregated human IgG was prepared by heating a

solution of 10 g/l purified human IgG at 63°C for 30 min.

ANIMAL EXPERIMENTS

Inbred male NMRI mice (40–45 g) were injected intravenously with 200 μl of one of the human sera under study. Forty microlitres of blood collected from the tail into heparinised capillaries was sampled for counting of PMN, mononuclear cells (MNC), and erythrocytes immediately before and at various intervals after the injection. Leucocyte counts were corrected for changes in erythrocyte counts. The effect of each serum was calculated as the mean of two experiments, which generally differed by less than 10%. The results for each of the patient groups are presented as mean $\pm$ SD of the calculated values for each of the individual sera.

STATISTICAL ANALYSIS

Statistical significances were determined by the Student's *t* test.

MICROSCOPIC STUDIES

Tissue specimens were taken from the lungs, kidneys, spleen, and liver of animals killed 12 min after being injected with 200 μl serum of patients with FS. Part of each of these specimens was snap frozen for immunohistochemical evaluation and part was processed for methyl methacrylate embedding, as described elsewhere,²² for histological examination. Human immunoglobulin was detected by the indirect two step immunoperoxidase technique with the use of antisera against human IgG (Dakopatts, Denmark).

Results

EFFECT OF PATIENT SERA

The course of the leucocyte counts made after the injection of 200 μl serum from FS patients into mice showed a drop for both PMN and MNC within three to six minutes after the injection (Fig. 1). The maximum decrease was found after nine to 12 minutes, the range amounting to 98–34% of the initial PMN count. Shortly after this peak, rebound granulocytosis occurred at about 21–24 minutes, whereas the number of MNC increased more gradually. Table 1 shows the C1qBA results for the patient sera and the changes in the numbers of circulating PMN 12 minutes after the injection of 200 μl serum. Correlation between these parameters was not found.

After the injection of 200 μl serum from RA patients without neutropenia the sera of two

Table 1 Clinical data of the present patients, the effect of injection of patient serum into mice, and the IgG/IgM concentrations of the fractionated serum pools under study

Patient No	Age (years)	Sex	Circ. PMN ×10 ⁶ /l	ClqBA* (mmol/l)	PMN response† (%)	Pool I‡		Pool II‡	
						IgG (g/l)	IgM (g/l)	IgG (g/l)	IgM (g/l)
<i>Felty's syndrome</i>									
1	55	M	350	840	71	14.0	0.2	0.2	2.6
2	58	F	70	900	63				
3	74	M	90	1580	69				
4	69	M	120	50	98				
5	71	F	200	1160	62	23.0	0.1	0.1	1.8
6	76	F	1140	110	47				
7	48	F	1360	41	41	7.0	0.1	0.1	1.4
8	81	F	1680	800	34				
<i>Rheumatoid arthritis</i>									
1	68	M	2840	180	65	17.5	0.3	0.1	2.0
2	73	F	3160	220	122				
3	54	M	3200	780	56	16.2	0.2	0.2	2.2
4	82	F	3650	490	110	12.8	0.2	0.1	1.8
5	59	F	4340	980	92				
6	66	F	4520	390	98				
Normal controls			2500-7000	<10					

*ClqBA=Clq binding assay.
†Number of circulating PMN 12 min after the injection of 200 µl serum of patients into mice. Values are the mean of two experiments, expressed as the percentage of PMN counts measured at t=0.
‡Pool I=serum fractions containing predominantly monomeric IgG; pool II=serum fraction containing complexed immunoglobulins of high molecular weight.

Fig. 1 Effect of intravenous injection of 200 μ l serum of patients with Felty's syndrome (FS) or rheumatoid arthritis (RA), serum of healthy controls (HC), and phosphate buffered saline (PBS), on the number of circulating PMN (solid lines) and mononuclear cells (dashed lines) in mice. The data are expressed as percentage of leucocyte counts measured at $t=0$ and are corrected for changes in erythrocyte counts. PBS was tested in three mice. n =number of human sera investigated.

patients led to a significant temporary decrease of the PMN and MNC counts and those of four patients only to a slight decrease or even an immediate increase (Fig. 1, Table 1). The difference between the maximum effect of FS and RA sera on the number of circulating PMN was statistically significant ($p=0.03$). Mice injected with 200 μ l HC serum always showed an immediate increase in the number of both circulating PMN and MNC (Fig. 1). Leucocyte counts did not change significantly after the injection of 200 μ l phosphate buffered saline (PBS, Fig. 1).

Increased vascular permeability, as judged from a rise of the erythrocyte counts, was not found for PBS or HC serum but was present nine minutes after injection of FS serum or heat aggregated IgG (AIGG) (Table 2).

Table 2 Course of erythrocyte counts determined at various intervals after the injection into mice of 200 μ l PBS, healthy control serum, serum of patients with Felty's syndrome, or 1 g/l heat aggregated IgG*

Time (min)	PBS†	HC serum (n=4)	FS serum (n=8)	AIGG†
0	100	100	100	100
9	96.3±1.5	89.3±4.5	106.8±5.2	111.2±10.6
21	90.6±2.1	87.0±1.0	98.6±4.7	105.0± 9.8
30	87.3±4.7	87.6±4.0	94.0±6.4	96.5± 6.0

*Data were expressed as the percentage of erythrocyte counts measured at $t=0$. n =number of human sera investigated.
†PBS and AIGG were tested in three mice each.

EFFECT OF IMMUNE COMPLEXES IN PATIENT SERA

The role of IC in the effect of FS serum in mice was investigated by PEG precipitation of the IC present in the sera of FS patients. The number of circulating PMN decreased at most to a mean of 60% of the initial PMN count after injection of untreated patient serum, whereas PMN counts did not drop after injection of sera treated with PEG (Fig. 2A). The number of circulating MNC decreased on average by 10% with non-treated sera and only increased with PEG treated sera (data not shown). In control experiments the effect of the injection of donor serum or PBS on the number of leucocytes was the same before and after PEG treatment of both (data not shown).

To find out whether the effect of IC on the number of circulating leucocytes is dose dependent we performed experiments with serial dilutions of heat aggregated human IgG. The results showed a decrease in the number of PMN and MNC dependent on concentration. Injection of 200 μ l AIGG at concentrations higher than 1 mg/ml led to death of the animal within five to 10 minutes. Similar concentrations of unaggregated IgG did not induce a decrease in the number of circulating PMN or MNC (see Fig. 2B for the PMN counts).

To find out whether the differences in the effect of sera from FS and RA patients on the number of circulating leucocytes could be attributed to differences in the concentrations of aggregated immunoglobulins in these sera we studied the effect of serum

fractions obtained by Sepharose 4B gel filtration. The serum fractions predominantly containing monomeric IgG and those containing complexed immunoglobulins of high molecular weight were pooled (pool I and pool II respectively). After the pools were concentrated to approximately 5 ml the IgG and IgM concentrations were determined (Table 1). Differences in the concentrations of IgG and IgM in pool II of the patients with FS or RA were small. The injection of 200 μ l of the 7S material (pool I) produced an increase in the number of circulating PMN and MNC in all samples tested. The injection of 200 μ l of material containing

complexed immunoglobulins (pool II) was followed by a distinct decrease of PMN and MNC counts with the three FS serum pools tested and a less pronounced decrease of the leucocyte counts with only two of the three RA serum pools (see Fig. 3 for the PMN counts).

LIGHT MICROSCOPY AND IMMUNOHISTOCHEMICAL EVALUATION

Light microscopical sections of lung, kidney, spleen, and liver tissue from mice injected with 200 μ l serum from patients with FS showed increased sequestration of PMN and MNC in the vascular bed and

Fig. 2 Effect of intravenous injections on the number of circulating PMN in mice. The data are expressed as percentage of leucocyte counts measured at $t=0$ after correction for erythrocyte counts. (A) Effect of injection of 200 μ l serum of patients with Felty's syndrome, before (solid lines) and after (dashed lines) incubation of the sera with PEG. n =number of patients investigated. (B) Effect of injection of 200 μ l of serial dilutions of a solution of 1 mg/ml (g/l) heat aggregated IgG (AIGG) and the effect of 1 mg/ml IgG. Each solution was tested in three mice.

Fig. 3 Effect on the number of circulating PMN in mice of intravenous injection of 200 μ l serum—pool I (dashed lines), pool II (solid lines)—obtained by Sepharose 4B gel filtration of serum of patients with Felty's syndrome (FS) and rheumatoid arthritis (RA). The pools were made from serum fractions containing monomeric immunoglobulins (pool I) and serum fractions containing immunoglobulins in fractions with higher molecular weight (pool II).

interstitium of the lungs compared with mice injected with PBS. Other organs did not show an increased number of leucocytes after the injection of patient serum.

Immunohistochemical studies showed the presence of human IgG, IgA, and IgM in the lung tissue of mice injected with patient serum, especially at places with aggregations of PMN. Negative results were obtained in mice injected with PBS.

Discussion

The present results show that serum from patients with FS and some patients with RA can induce leucopenia (primarily neutropenia) when injected into mice. Leucocyte sequestration was found in the vascular bed of the lungs. Evidence is provided that this leucopenia is caused by IC present in these sera.

Administration of soluble IC to small rodents provides a relatively simple basis for assessment of the effects of circulating IC on the kinetics of leucocytes. The use of heterologous immunoglobulins is an acceptable model because there is known to be a high degree of cross reactivity in binding between complexed human IgG and animal leucocytes.^{17 23 24} A fall in the number of circulating PMN and leucocyte aggregation in pulmonary vessels after intravenous administration of IC in animals, as found in the present study, has already been described in reports of early studies on anaphylaxis.^{25 26} The mechanisms underlying this effect of IC on PMN in animals have been studied by several authors. Neutropenia follows intravascular PMN aggregation and increased adherence of PMN to endothelial cells.^{27 28} This increased adherence has been related to receptor mediated binding of IC to PMN and to complement activation, including C5a anaphylatoxin generation.^{18 29-31} The latter may induce release of mediators by PMN and changes in the composition of glycoproteins in the PMN membrane that increase PMN adhesiveness.^{18 32-35}

Serum fractions from FS patients that contained IC were more active in producing leucopenia than corresponding fractions from RA patients. This difference may be due to differences in the concentration of IC since a concentration dependent decrease in the number of PMN was found after injection of serial dilutions of AIGG, but this is not supported by the absence of correlation between the leukokinetic effect of the patient sera and either the C1qBA results or the immunoglobulin concentrations of the serum fractions tested. Differences in the size of IC in sera, such as have been found between patients with FS and RA,³⁶ might be an

alternative explanation, but the composition and the electric charge of the IC and their ability to activate complement and to bind to PMN may also be of influence. Further work is needed to clarify this point.

Neutropenia has been thought to be caused by circulating IC in cases of chronic idiopathic neutropenia³⁷ and SLE.³⁸ In vitro studies have shown that serum from patients with FS can induce PMN aggregation and increased adherence of PMN to endothelial cells.³⁹ Moreover, Calabresi *et al* saw a transient neutropenia develop in healthy volunteers immediately after injection of serum from two FS patients,⁴⁰ and this pattern was similar to that of the PMN kinetics in mice observed in the present study, which suggests that in man, too, this effect of FS serum was due to IC.

There is thus evidence supporting the concept that the interaction between PMN and IC in the peripheral blood depresses PMN counts in experimental and human immune complex disease. Whether neutropenia develops in RA patients with circulating IC may depend on the granulopoietic activity in the bone marrow, the size of the spleen, and the amount and type of the IC. The results of this study show that in patients with FS circulating IC have a specific potential for the induction of peripheral sequestration of PMN in mice. This, in combination with the fact that most FS patients have high levels of circulating IC^{36 41} and neutropenia due to a shift of PMN from a circulating to a marginated pool,⁵ seems to suggest that the persistent in vivo interaction between IC and PMN in FS has a previously neglected role in the pathogenesis of the disease.

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