Phenotypic and genotypic analysis of mononuclear cells from patients with Felty's syndrome

M J Ahern, P J Roberts-Thomson, J Bradley, C Story, P Seshadri

Abstract

Phenotypic and genotypic characteristics of the peripheral blood mononuclear cells in nine patients with Felty's syndrome have been examined. One patient had an increased number and percentage of peripheral blood mononuclear cells with the phenotype CD3+ Leu-7+ CD16+ and showed a clonal rearrangement of the T cell receptor B chain gene. The remaining eight patients all showed a germline configuration of the T cell receptor B chain gene. In two patients an increased proportion of CD3+ Leu-7+ CD16− peripheral blood mononuclear cells (45 (SD 11)% of peripheral blood mononuclear cells) were found, while the remaining six patients had proportions of CD3+ Leu-7+ cells similar to those of patients with uncomplicated rheumatoid arthritis. These data confirm that patients with Felty's syndrome are heterogeneous, with at least three different peripheral blood mononuclear cell phenotypic subsets. One subset is characterised by a clonal expansion of an unusual lymphocyte subpopulation, another by polyclonal expansion, and the third subset has the same proportions of peripheral blood mononuclear cells as patients with uncomplicated rheumatoid arthritis.

Felty's syndrome is defined as rheumatoid arthritis, splenomegaly, and neutropenia (neutrophils $<2\times10^9$/l).[1] Implicit in this definition is the exclusion of patients in whom the neutropenia and splenomegaly are attributed to drug reactions, malignancy, hepatic cirrhosis, or amyloidosis.

A group of patients has been described whose blood, bone marrow, and spleen contained an increased proportion of T cells with defined cell surface markers and a characteristic morphology.[2 3] These T cells have the characteristics of large granular lymphocytes with eccentric nuclei and abundant pale blue cytoplasm containing many azurophilic granules, and often express surface markers defined by anti-CD8, anti-Leu-7, and anti-CD16 monoclonal antibodies.[3 4] The increase of this lymphocyte subset could be due to an expansion of an abnormal T cell clone or, alternatively, due to a polyclonal proliferation of lymphocytes with a homogeneous phenotype. Patients with this lymphocyte subset are also often neutropenic,[3 4] and some have cytogenetic abnormalities, which have been characterised as a clonal rearrangement involving the T cell antigen receptor β genes.[5 6] As rearrangements of the T cell antigen receptor β gene have provided a marker of the clonal nature of chronic T cell leukaemias and related disorders[5 6] these patients can be considered to have large granular lymphocyte leukaemia.

It should be noted that the clonality of T cell populations may be assessed by analysis of genes encoding the T cell antigen receptor. The T cell antigen receptor gene complex consists of α, β, and γ chains that rearrange in mature T cells.

Some of these patients with large granular lymphocyte leukaemia have splenomegaly and rheumatoid arthritis and hence fufil the criteria established for Felty's syndrome. As mechanisms of the neutropenia, prognosis, and management may differ between the two conditions it is important to characterise these patients further. We determined, therefore, the phenotypes of blood lymphocytes from nine patients with Felty's syndrome, and assessed the clonality of their lymphocytes.

Patients and methods

PATIENTS

Nine patients fulfilled the criteria for Felty's syndrome. They had a combination of classical rheumatoid arthritis according to American Rheumatism Association criteria,[7] sustained neutropenia ($<2{\cdot}0\times10^9$/l) persisting for a minimum of 12 months, and splenomegaly. The patients were treated with a variety of non-steroidal anti-inflammatory drugs and disease modifying agents (methotrexate (one patient, auranofin (two)). No patient was taking oral or systemic corticosteroids.

The control group comprised 58 patients with uncomplicated rheumatoid arthritis—that is, no neutropenia—with a mean age of 66 years (range 43–82).

CELL SURFACE MARKERS

Peripheral blood mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation.[8] The expression of cell surface antigen was studied using a panel of fluorescein conjugated or phycoerythrin conjugated monoclonal antibodies. Table 1 describes the antigens recognised by these monoclonal antibodies, according to the 3rd international workshop and conference on human leucocyte differentiation antigens.[9]

For flow cytometry 5×10^5 cells were incubated with the specific monoclonal antibody or with a control murine myeloma followed by fluorescein conjugated goat antimouse immunoglobulin.

Department of Immunology/ Rheumatology, Flinders Medical Centre, Adelaide, Australia
M J Ahern
P J Roberts-Thomson
J Bradley

Department of Haematology, Flinders Medical Centre and RGH, Daw Park, Adelaide, Australia
C Story
P Seshadri

Correspondence to:
Dr M J Ahern, Department of Medicine, Flinders Medical Centre, Bedford Park, SA 5042, Adelaide, Australia.

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Table 1: Monoclonal antibodies and cluster determination

Monoclonal antibody		Cluster determination
Anti-Leu-2a	(T suppressor/cytotoxic cells)	CD8
Anti-Leu-3a	(T helper/inducer cells)	CD4
Anti-Leu-4	(pan T)	CD3
Anti-Leu-7	(subset of large granular lymphocytes)	
Anti-Leu-11	(Fc receptor for IgG)	CD16
Anti-OKT11	(sheep erythrocyte receptor)	CD2
Anti-OKM1	(CR3)	CD11b

For two colour fluorescein studies the unlabelled monoclonal antibody (anti-Leu-7, anti-Leu-11) was added first, followed by the fluorescein conjugate, then a blocking step using purified mouse immunoglobulin (5 µl at 1 mg/ml) was carried out, and, finally, the phycoerythrin conjugated monoclonal antibody (anti-Leu-4, anti-Leu-7) was added. Incubation steps were performed on ice to prevent capping. Cells were analysed by a FACS IV flow cytometer (Becton Dickinson).

GENOMIC BLOT HYBRIDISATION

High molecular weight genomic DNA free of protein was obtained by standard procedures[10] from peripheral blood lymphocytes separated on Lymphoprep (Nycomed, Oslo, Norway). In addition, DNA was isolated from purified blood neutrophils from patient 1. DNA, 10 µg from each patient, was digested with the restriction enzymes Eco R1, Bam H1, and Hind 111 (Pharmacia LKB Biotechnology), electrophoresed through 0·7% agarose, and transferred to Gene Screen Plus hybridisation transfer membrane (Du Pont, Australia) by the method of Southern.[11] Restricted DNA gene fragments were identified by their hybridisation with the T cell receptor B chain (T_B) gene probe, nick translated to a specific activity of 2×10^8 cpm/µg DNA with ^{32}P labelled nucleotides. After hybridisation, stringent washing of filters was carried out and specific hybridisation visualised as bands on *x* ray film.[10]

Results

Tables 2, 3, and 4 show the clinical and haematological features of the patients studied. All had splenomegaly. There was no increase in large granular lymphocytes in the peripheral blood examined on several occasions. Bone marrow examination of all patients by two experienced haematologists showed no increase in cells with the morphology of large granular lymphocytes (<5%). In patient 1 four bone marrow examinations were performed at 12 month intervals and no increase in the number of large granular lymphocytes was seen.

Patients 1, 2, 4, and 7 had an increased percentage of CD8 positive lymphocytes and an increased percentage of cells expressing Leu-7 (table 3). Only patient 1 had an increased

Table 4: Two colour immunofluorescence

Patient No	% Positive PBMC*	
	CD3-Leu-7	CD3-CD16
1	38	41
2	21	2
3	11	3
4	37	7
5	14	3
6	8	ND
7	52	6
8	ND*	2
9	18	2
RA* controls (n=5) Mean (SD)	19 (2·2)	2 (1)

*PBMC=peripheral blood mononuclear cells; RA=rheumatoid arthritis; ND=not done.

Table 2: Clinical features of patients with Felty's syndrome

Patient No	Age	Sex	Duration of RA* before neutropenia	Nodules	RF*† (IU/ml)	Erosions	ANA* titre
1	68	M	Simultaneous	Present	1880	No	1/160
2	75	M	9	Absent	85	Yes	1/40
3	76	F	13	Present	1740	Yes	1/640
4	62	M	0·1	Present	4896	Yes	1/160
5	64	F	0·1	Present	3744	Yes	Neg
6	68	F	7	Present	1190	Yes	1/2560
7	64	M	35	Present	5220	Yes	1/640
8	73	F	5	Present	13428	Yes	1/2560
9	51	F	17	Present	9324	Yes	1/1280

*RA=rheumatoid arthritis; RF=rheumatoid factor; ANA=antinuclear antibody.
†RF: normal <60 IU/ml.

Table 3: Haematological features of patients with Felty's syndrome

Patient No	WCC* ($\times10^9$/l)	Lymphocytes ($\times10^9$/l)	Neutrophils ($\times10^9$/l)	% Positive PBMC*						
				CD3	CD4	CD8	Leu-7	CD16	CD2	CD11
1	2·0	1·50	0·14	94	17	77	42	44	88	23
2	3·2	2·56	0·42	98	12	89	32	10	88	15
3	2·6	0·65	1·53	78	65	20	15	15	80	ND*
4	2·5	1·33	0·75	88	42	44	42	19	81	ND
5	1·3	0·91	0·20	76	49	22	23	12	89	ND
6	1·4	0·96	0·28	68	44	21	12	16	82	ND
7	3·2	1·37	1·47	96	25	64	64	13	70	16
8	1·9	1·13	0·66	82	44	30	30	14	89	ND
9	3·3	1·12	1·68	74	47	23	29	14	89	14
RA* controls (n=58)				73	48	29	26	15	ND	16
SEM				1·5	1·4	1·4	3·4	2·9	—	1·3

*WCC=white cell count; PBMC=peripheral blood mononuclear cells; RA=rheumatoid arthritis; ND=not done.

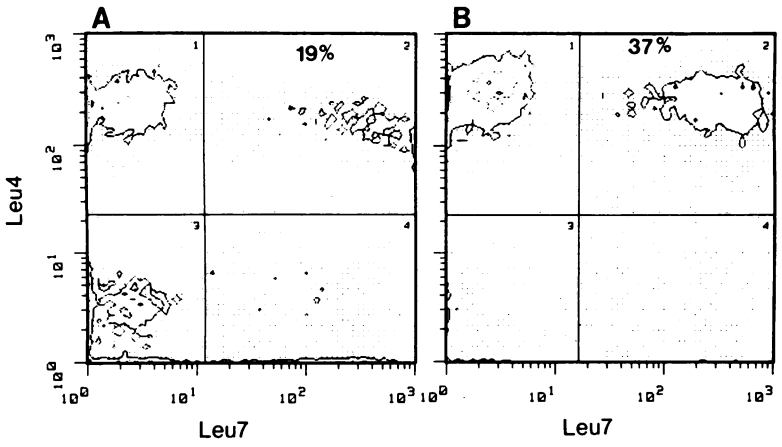

Figure 1: Two colour immunofluorescence flow cytometry of Leu-4 (CD3) and Leu-7 expression on peripheral blood mononuclear cells from patient 4. The figures show phycoerythrin conjugated anti-CD3 (Leu-4) monoclonal antibody (red) versus fluorescein conjugated anti-Leu-7. The x axis represents the log of green fluorescence and the y axis the log of red fluorescence. (A) Control with uncomplicated rheumatoid arthritis; (B) patient 4 with Felty's syndrome.

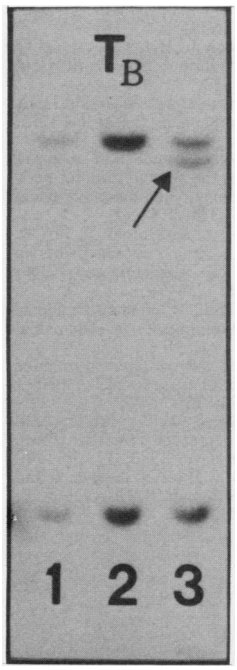

Figure 3: T cell receptor B chain (T_B) analysis of DNA from patient 1 digested with Eco RI. Lane 1 shows the unrearranged T_B hybridisation pattern obtained with normal peripheral blood lymphocyte DNA. Lanes 2 and 3 show T_B patterns in patient 1 neutrophil and lymphocyte DNA respectively isolated from the same peripheral blood sample. The arrow denotes the position of the rearranged band.

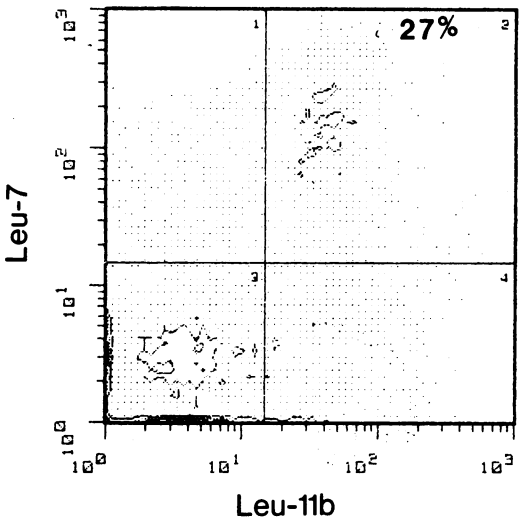

Figure 2: Two colour immunofluorescence flow cytometry of Leu-7 and CD-16 (Leu-11b) expression on peripheral blood mononuclear cells from patient 1. The figure shows phycoerythrin conjugated anti-Leu-7 (red) versus fluorescein conjugated anti-CD16 (Leu-11b). An increased proportion of cells coexpressing CD16, Leu-7 is seen.

absolute number and percentage of CD16 positive cells.

Two colour immunofluorescence of mononuclear cells from patient 1 confirmed that CD3+ cells coexpressed Leu-7 and CD16 at percentages that were greater than normal (table 4). Also, two colour immunofluorescence of peripheral blood mononuclear cells from patients 4 and 7 confirmed the increased proportion of cells coexpressing CD3+ Leu-7+.

Figure 1B shows the increased percentage of cells coexpressing CD3 (leu-4) and Leu-7 in patient 4 compared with a control patient with uncomplicated rheumatoid arthritis (fig 1A).

Figure 2 shows the two colour immunofluorescence of mononuclear cells from patient 1 coexpressing Leu-7 and CD16 (Leu-11b) at levels greatly increased above normal. Of the cells that were Leu-7 positive, 72% coexpressed Leu-11b.

REARRANGEMENT OF THE T CELL RECEPTOR B CHAIN (T_B) GENE

To detect the presence of any predominant clones of T lymphocytes in the peripheral circulation of the nine patients with Felty's syndrome, DNA from their lymphocytes was analysed with the T_B gene probe. In eight patients no T_B gene rearrangements were detectable, indicating that in these patients there was less than 2% of T lymphocyte clones in the total lymphocyte population. In patient 1 DNA restricted with Eco R1, a band of approximately 10 kb was present, in addition to the germline bands of 4·0 and 11·0 kb (fig 3, lane 3 compared with lane 1). No new bands were detected when this DNA was digested with Bam H1 and Hind 111.

Discussion

We have shown that one of nine patients with Felty's syndrome had a clonal proliferation of lymphocytes with the same phenotypic expression as large granular lymphocytes. There was no increase in the number of large granular lymphocytes in the peripheral blood or bone marrow, however, as independently confirmed by two experienced haematologists. Two colour immunofluorescence showed a marked increase in CD3+ CD8+ Leu-7+ CD16+ cells in this patient, a phenotype rarely seen in normal peripheral blood lymphocytes. Three other patients with Felty's syndrome had increased percentages of CD8+ cells: only patients 4 and 7 showed an increased proportion of CD3+ Leu-7+ cells, but CD3+ CD16+ cells were normal or only slightly increased.

There was evidence of T_B gene rearrangement of DNA isolated from the lymphocytes of patient 1 but no evidence in the remaining eight patients. A polyclonal population of normal T cells has numerous different T_B gene rearrangements. Multiple differently sized T_B gene rearrangements result in DNA fragments after restriction enzyme digestion. Southern blot analysis shows no single rearranged band, other than those bands representing the germline (unrearranged) form of the gene, because each rearrangement is below the threshold of sensitivity. A clonal expansion of T lymphocytes has a unique DNA rearrangement pattern, however, that can be seen as a distinct band. Southern blot analysis can detect clonal proliferation of lymphocytes comprising as little as 2% of the total cell population in the sample.[12]

The new band in patient 1 indicated the presence of a unique T cell clone. The absence of this new band in the neutrophil DNA sample of patient 1 (fig 3, lane 2) prepared at the same time as the corresponding lymphocyte DNA sample indicated that the band was not the result of polymorphism or partial digestion.

Our study reports findings similar to those of Freimark *et al*[13] and Loughran *et al*,[14] who showed that mononuclear cells from patients with large granular lymphocyte leukaemia expressed the phenotype CD8+ Leu-7+ CD16+ and found a clonal rearrangement of the T_B gene; in contrast, patients with Felty's syndrome had no T_B gene rearrangement. As in

Freimark's study, we showed an identical additional band of 10·0 kb on Southern blot analysis after digestion with Eco R1.

Patient 1 had a clonal expansion of lymphocytes with phenotypic characteristics shared with large granular lymphocytes. It can be argued that this patient had large granular lymphocyte leukaemia; no increase in morphologically defined large granular lymphocytes was seen in the peripheral blood or bone marrow, however. In contrast with the remaining eight patients with Felty's syndrome, the neutropenia in this patient coincided with the onset of his arthritis. The remaining eight patients had normal neutrophil counts at the onset of their arthritis with two patients (4 and 5) becoming neutropenic within one month. Patient 1 has developed typical rheumatoid nodules with no joint erosions. On no occasion has he shown a blood or marrow lymphocytosis. His arthritis and neutropenia have responded to low dose weekly methotrexate (12·5 mg). In this patient there were no clinical responses to corticosteroids and gold treatment, nor was there an increase in the neutrophil count.

The proposed mechanisms of the neutropenia in Felty's syndrome are numerous, with evidence confirming that cellular and humoral immune mechanisms may be present in different subsets of patients with Felty's syndrome.[15 16] Phenotypic analysis of mononuclear cells from our patients has allowed us to identify three subsets of patients with Felty's syndrome: (*a*) a patient with a clonal expansion of an unusual subset of lymphocytes (CD3+ CD8+ Leu-7+ CD16+)—possibly equivalent to large granular lymphocyte leukaemia; (*b*) two patients with a polyclonal increase of a subset of lymphocytes (CD3+ CD8+ Leu-7+ CD16−); (*c*) six patients with normal numbers of mononuclear cells expressing Leu-7 and CD16.

The patients with classical Felty's syndrome studied by Linch *et al*[2] and Loughran *et al*[14] had normal numbers of peripheral blood mononuclear cells expressing Leu-7 and CD8. Freimark *et al*, however, found heterogeneity in the phenotype and found subsets similar to our findings.[13] It will be necessary to correlate these defined phenotypic subsets with functional mechanisms either of a humoral or cellular nature. This work is currently in progress.

Our study confirms the importance of phenotypic and genotypic analysis in the assessment of patients with Felty's syndrome as they seem to be a heterogeneous group. Characterisation of this heterogeneity might lead to better definition of mechanisms of the neutropenia, resulting in more rational therapeutic approaches.

The cDNA clone Jurkat-2[17] for the B chain of the T cell receptor was generously donated by Dr Tak Mak (Ontario Cancer Institute, Toronto, Ontario, Canada).

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