Broadening of the T cell receptor spectrum among rheumatoid arthritis synovial cell-lines in relation to disease duration

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Abstract

Objective

The aim of the study was to evaluate the T cell receptor (TCR) family usage in T cell-lines from sub-cutaneous nodules and synovium from patients with rheumatoid arthritis (RA), with specific reference to the duration of symptoms. In vitro adherence characteristics of nodular T cells was studied as well.

Methods

Monoclonal antibodies were used to determine the distribution of TCR families in T cell-lines from synovium of patients with early and long-standing RA, from rheumatoid nodules and control tissues. An in vitro binding assay with T cell-lines from 2 rheumatoid nodules was performed.

Results

In early RA synovium, a restricted TCR family usage was observed in 5 out of 8 patients, contrary to long-standing disease, peripheral blood, ileum and colon. In RA nodules, a similar degree of restriction was noted. Moreover, the same TCR family was overexpressed by T cell-lines from different nodules derived from the same patient. T cell-lines from rheumatoid nodules demonstrated a preferential in vitro adherence to rheumatoid synovium and rheumatoid nodules, while no binding was observed on skin or tonsil.

Conclusion

The TCR spectrum among RA synovial cell-lines broadens in relation to the disease duration. The overexpression of the same TCR family in different rheumatoid nodules from the same patients, and the in vitro adherence of T cell-lines from rheumatoid nodules may be indicative for recirculation between the different disease manifestations in RA.

Key words

T cell receptor, rheumatoid arthritis, rheumatoid nodules, rheumatoid synovium.

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**The contributions of both authors were equal; therefore they should both be considered as first authors.*

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease mainly characterized by a destructive polyarthritis. Although the pathogenesis is still largely unknown, T cells are considered to play a role at some point in this disease based on the strong MHC class II-linkage of the disease, the ability to transfer the disease by T cells in experimental models of arthritis, the predominance of T cells in lymphoid infiltrates at the primary sites of inflammation, and the beneficial effect of some T cell directed therapies (1-5). An interesting three-step model for the initiation and perpetuation of RA was recently proposed, in order to conceal the contribution of dendritic cells and T cells in the pathogenesis of this disease (6). According to these authors, an initial phase characterized by maturation of dendritic cells is followed by a T cell-dependent phase that may be necessary for the induction of a chronic disease with joint destruction and extra-articular features. Finally, a chronic phase is proposed in which a polycellular dysregulation occurs, ultimately leading to a synovial tissue autonomy.

The clinical spectrum of RA includes articular as well as extra-articular manifestations, such as the occurrence of rheumatoid nodules, pleuritis, pericarditis, episcleritis and vasculitic skin lesions. Rheumatoid nodules are granuloma-like formations, consisting of a central zone of fibrinoid necrosis surrounded by a pallissading layer of histiocytes and an outer zone of lymphoid aggregates, organized around small vessels (7-9). The precise immunological relationship between rheumatoid nodules and rheumatoid synovium is as yet unknown.

In this paper we describe the spectrum of TCR family usage among interleukin-2 (IL-2)-dependent T cell-lines from rheumatoid nodules and rheumatoid synovium. We specifically looked for different patterns according to the duration of symptoms. T cell-lines from rheumatoid nodules were further analysed for *in vitro* binding characteristics. We previously demonstrated that IL-2-dependent T cell-lines from rheumatoid nodules display restricted TCR usage, with CDR3 motifs that are best explained by antigen-driven *in situ* activation (10, 11).

Patients and methods

Patients and samples

27 rheumatoid nodules were obtained from 19 patients with classical RA according to the ACR criteria (12). Rheumatoid synovium was obtained from 7 RA patients with long-standing (>5 years disease duration and >1 previous disease modifying antirheumatic drug [DMARD] treatment) and 8 from patients with early disease (< 1 year of symptoms and no previous DMARD) on the occasion of knee joint replacement for destructive arthritis or by needle arthroscopy of the knee. In one patient, additional synovial tissue from the hip was obtained as well. The clinical and serological data of the RA patients from whom rheumatoid nodules and synovium were obtained are summarised in Table I.

Peripheral blood lymphocytes (PBL) were derived from 6 RA patients. Ileum and colon mucosal biopsies were obtained from 10 non-inflammatory controls and 21 patients with spondyloarthropathy, among whom 11 had inflammatory bowel disease. This study was approved by the Ethical Committee of our local Faculty of Medicine.

Needle arthroscopy

Synovial tissue samples were obtained under arthroscopic guidance from inflamed knee (swollen joint), using a fiber optic 1.8 mm arthroscope (Optical Catheter System™, Medical Dynamics Inc., Englewood, Co). All procedures were performed under local anaesthesia in an ambulatory setting. Sampling error was minimalized by obtaining 8-10 samples from macroscopically inflamed synovial areas (suprapatellar pouch) within the joint.

T lymphocyte expansion

T cell-lines were expanded as reported previously (10). Briefly, small tissue fragments from rheumatoid nodules, rheumatoid synovium, ileum or colon (2 to 5 mm^3) were transferred into separate wells on a 24-well culture plate (Gibco, Grand Island, NY) and incubated in RPMI medium (Gibco) containing recombinant IL-2 (50 U/ml; Eurogenetics, Belgium), 10% autologous serum, antibiotics (10 U/ml Penicillin-G, 10 U/ml streptomycin sulfate and 0.025 µg/ml

amphotericin B) and 0.5% L-glutamine. For generation of T cell-lines from the gut, gentamycin (50 µg/ml) was added to the culture medium.

A total of 89 lymphocyte cell-lines from rheumatoid nodules, 30 from rheumatoid synovium, 44 from ileum and 31 from colon were obtained for flowcytometric analysis. Peripheral blood lymphocytes were first isolated by Ficoll-Hypaque separation (Pharmacia, Upsala, Sweden) and then kept under the same conditions as the tissue-derived lymphocytes.

Flow cytometry

The analysis of TCR family expression was performed by flow cytometry with double labeling: phyco-erythrin-labeled anti-CD3 antibodies (Leu4, clone SK7; Becton Dickinson, San Jose, CA, USA) and fluorescein-labeled anti-αβ TCR family antibodies (T Cell Diagnostics, Cambridge, MA). The following anti-TCR antibodies were used: anti-Vβ5A, -Vβ5B, -Vβ5C, -Vβ6, -Vβ8, -Vβ12 en Vα2, covering approximately 20-25% of the Vβ TCR family repertoire. αβ and γδ TCR expression were studied using anti-pan αβ (clone BMA031, Immunotech, Marseille, France) and γδ TCR antibodies (clone 5A6.E9, T Cell Diagnostics). Staining with anti-CD4 (Leu3a, clone SK3; Becton Dickinson) and anti-CD8 (Leu2a, clone SK1; Becton Dickinson) was performed as well. Routinely, 10,000 events from each sample were collected.

Acquired data were analysed using LYSIS II® and Attractors® software (Becton Dickinson).

Table I. Clinical data from RA patients from whom rheumatoid nodules (1-19) or synovium (20-34) were derived.

Patient	Sex	Age	RF	Duration	DMARD	DRB1 typing
1	M	47	+	10	DP	0101/0401
2	M	72	+	9	LEVA	0101/0701
3	M	71	+	43	MTX	0401/0101
4	F	78	+	50	Nonz	0301/15
5	M	69	+	1	None	ND
6	M	62	+	1	Gold	ND
7	M	57	+	20	DP	ND
8	F	64	+	44	MTX	ND
9	M	71	+	10	MTX	0101/1301
10	F	72	+	18	SASP	ND
11	M	64	+	10	MTX	ND
12	M	48	+	2	None	0801/15
13	M	68	+	8	None	ND
14	M	69	+	20	MTX	ND
15	M	65	+	25	MTX	ND
16	F	64	+	17	MTX	ND
17	F	51	+	20	MTX	0401/0408
18	F	60	+	29	MTX	0101/1301
19	F	49	+	5	None	1001/1102
20	F	72	+	18	SASP	ND
21	M	71	+	22	LEVA	ND
22	F	53	+	6	MTX	ND
23	F	38	+	22	MTX	ND
24	M	44	+	13	None	ND
25	F	54	+	10	None	ND
26	F	51	+	20	MTX	0401/0408
27	M	46	-	0.4	None	ND
28	F	72	+	0.4	None	ND
29	F	67	-	0.3	None	0404/0408
30	F	34	+	0.4	None	0401/0301
31	M	40	-	0.3	None	0401/1501
32	F	43	-	0.8	None	04
33	F	51	-	0.4	None	ND
34	F	63	-	0.6	None	0101/04

Sex (Male/Female), Age (years), rheumatoid factor (RF), disease duration (years), disease modifying antirheumatic drugs (DMARD) at the moment of tissue sampling: methotrexate (MTX), D-penicillamin (DP), levamisole (LEVA), sulphasalasin (SASP), and gold salts. ND: not done.

In vitro *binding assay*

The *in vitro* adherence of T cell-lines from 2 rheumatoid nodules derived from 2 RA patients was performed using a modified Stamper-Woodruff assay (13). Briefly, T cell-lines were incubated with anti-CD45RO-EPOS (UCHL-1, Dako, Glostrup, Denmark), for 45 min at 4°C, according to the manufacturer's instructions, and kept at 4°C until the assay was performed. Freshly frozen tissue sections (5 µm) of rheumatoid nodules, rheumatoid synovium and control tissues such as tonsils and skin were then overlayed with the pre-labeled cells at a concentration of 500,000 cells from each tissue section and incubated subsequently for 30 min at 4°C. From each investigated source, 3 different sections were overlayed with labeled cells. After incubation, the non-adherent cells were removed by rinsing in PBS (Gibco) for 20 minutes. Finally, the antibody-EPOS complex was visualised by adding the chromogen aminoethylcarbazole (AEC, Dako), according to the instructions, and mounted in aqueous medium (Mounting medium, Merck, Darmstadt, Germany). *In vitro* binding of T cells was quantified using a computerized image analysis system (Quantimet 500MC, Leica, Cambridge, UK): the results were expressed as the number of cells adhering/ mm^2 tissue.

Analysis of data

Restricted TCR family usage was defined as a > 30% expression of one of the tested TCR families (± a 10-fold increase in relative representation). Data were analysed using the χ^2 test with Yates' correction. $p < 0.05$ was considered as statistically significant.

Table II. Relative number of T cell-lines with restricted T cell receptor (TCR) family usage in T cell-lines from rheumatoid nodules, RA synovium, and control cell-lines.

	No. of pts. from whom tissue was obtained	Total no. of T-cell lines available for analysis	No. of T-cell lines with restricted TCR family usage	No. of pts. with at least one T cell-line characterized by a restricted TCR family usage	Relative no. of T-cell lines with restricted TCR family usage
Rheumatoid nodules	19	89	28	16	28/89 (31%)
Early RA synovium	8	16	7	5	7/16 (44%)
Late RA synovium	7	14	0	0	0/14 (0%)
Ileum	25	44	2	2	2/44 (5%)
Colon	11	31	0	0	0/31 (0%)
RA PBL	6	6	0	0	0/6 (0%)

Results

TCR expression among T cell-lines from rheumatoid nodules

A total of 89 T cell-lines were generated from 27 rheumatoid nodules derived from 19 RA patients. These T cell-lines carried CD4 (mean % CD4+/CD3+ cells: 88.0% ± 3.4) and the αβ TCR (mean: 96.1% ± 1.1). A restricted TCR family usage was observed in 28 T cell-lines from 16 patients (Table II). T cell-lines derived from one rheumatoid nodule or from different nodules in an individual patient tended to overexpress the same TCR family, even if the nodules were obtained with time interval. The TCR families overexpressed, however, differed from patient to patient. This phenomenon was not related to the disease duration, nor to the time interval between appearance and removal of the nodules.

TCR expression among cell-lines from rheumatoid synovium.

The results of TCR family usage among T cell-lines from rheumatoid synovium are shown in Table III. Fourteen T cell-lines from 7 patients with longstanding disease were generated. Clear restriction in TCR family usage could not be observed. In contrast, restricted TCR family usage among synovial T cell-lines was abundant in synovial T cell lines from early RA (7 out of 16 cell-lines originating from 5 patients). Thus, a significantly higher frequency of restricted TCR family usage was noted in early RA synovium compared to longstanding RA ($p < 0.05$). This could not be attributed to differences in CD4/CD8 T cell distribution as the majority of T cell-lines in both groups were CD4+ T cell-lines (mean % CD4+/CD3+ cells in early RA:

Table III. TCR family distribution in T cell-lines derived from rheumatoid synovium in long-standing RA (patients 1 - 7) and early RA (patients 8 - 15). The results are expressed as a percentage of the total CD3-positive cell population.

Patient	Cell-line	Vβ5A	Vβ5B	Vβ5C	Vβ6	Vβ8	Vβ12	Vα2
RA 1	B3	0	0	2	8	2	2	0
RA 2	C3	0	0	18	0	1	0	1
	C4	2	1	2	1	28	1	3
RA 3	B2	9	12	2	4	4	0	0
	C2	6	1	0	0	1	0	1
RA 4	B2	2	1	0	4	1	1	1
RA 5	Hip B2	10	11	2	4	5	1	2
	Hip B3	7	4	4	6	6	4	6
	Hip B4	13	13	3	5	5	1	4
	Knee B2	9	6	3	13	3	2	1
	Knee B3	5	3	1	2	3	2	3
	Knee B4	1	0	0	0	1	0	0
RA 6	B2	1	1	1	0	1	0	1
RA 7	B3	ND	2	2	1	3	0	1
RA 8	C2	0	0	11	1	0	0	1
RA 9	B2	2	0	31	1	1	4	5
	B3	2	2	2	3	2	5	21
RA 10	C2	0	1	1	3	2	0	4
	C3	1	0	1	1	4	1	1
	C4	0	0	1	1	2	3	1
	C5	1	1	0	2	2	1	99
RA 11	B4	1	1	29	1	3	1	46
	C2	4	2	1	1	6	1	3
RA 12	C4	0	0	0	0	0	0	0
RA 13	B2	27	0	0	0	0	0	0
RA 14	B2	0	0	0	93	0	0	91
	B3	0	0	43	0	10	0	6
RA 15	B2	0	1	0	1	96	5	1
	C2	0	0	0	7	1	0	0
	C3	0	1	0	6	2	0	84

84.6% ± 8.0, versus 84.6% ± 10.9 in late RA).

Among the TCR families over-represented in early RA synovial T cell-lines, the Vα2 TCR family was most prominently overexpressed: 4 cell-lines from 4 different early RA patients showed a positive bias in Vα2 usage. A representative example of a CD4+ synovial T-cell-line with preferential usage of the Vα2 and Vβ6 TCR family is shown in Figure 1.

TCR expression in control cell-lines (T cell-lines from peripheral blood, ileum and colon)

Contrary to the marked restriction in TCR family usage in T cell-lines from rheumatoid nodules and early RA synovium, T cell-lines from peripheral blood from RA patients showed a normal TCR family usage. Other control experiments consisted of tissue-derived T cell-lines from ileum and colon from patients with spondyloarthropathy (SpA) or non-inflammatory controls. These cell-lines had a mixed CD4/CD8 phenotype (47.9% ± 6.4 CD4 cells in ileum, and 51.8% ± 6.3 in colon). Of the 44 T cell-lines from ileum, only 2 cell-lines from 2 different patients displayed a restricted TCR family usage. Restricted TCR family usage was not observed in any of the 31 cell-lines from colon (Table II).

In vitro *adherence of T cell-lines from rheumatoid nodules*

An *in vitro* binding-assay was performed with T cell-lines from rheumatoid nodules from 2 RA patients (patients DKL and PJ). These cell-lines had a clear restriction in TCR family usage. The results of the *in vitro* adherence assay are shown in Figure 2. The relative fraction of cells that bound to the tissue substrate varies in rheumatoid synovium from 0.108°/₀₀ to 2.98 °/₀₀ and in rheumatoid nodules from 1.08°/₀₀ to 2.57°/₀₀. There was no difference in binding to an autologous or heterologous substrate. As the fraction of cells bound is also function of the surface of the tissue substrate, the results of the *in vitro* adherence assays were expressed as the number of cells bound/mm^2.

T cell-lines from rheumatoid nodules bind to rheumatoid nodules and RA synovium, but not to control tissues such as skin or tonsil. Interestingly, expanded T cell-lines from rheumatoid nodules showed a preferential adhesion to RA synovium, compared to subcutaneous nodules. Moreover, under the present experimental conditions, control cell-lines from inflamed bowel of patients with inflammatory bowel disease did not adhere to RA synovium (data not shown).

Discussion

This study focuses on the TCR family usage among IL-2 dependent T cell-lines from rheumatoid nodules, synovium and control tissues. The aim of the present study was to evaluate TCR family usage in RA synovial T cell-lines in relation with disease duration and to compare the TCR spectrum among these T cell-lines with cell-lines from rheumatoid nodules and other origins. Also, we analysed the *in vitro* T cell cross-adherence between rheumatoid synovium and rheumatoid nodules.

In the present study, we confirm the earlier and more preliminary finding of a marked oligoclonality among IL-2 expanded T cell-lines from rheumatoid nodules (10, 11). This finding occurs in almost all subjects studied, irrespective of the installed treatment and even in those patients without disease modifying antirheumatic drugs. The predominant TCR family varies from patient to

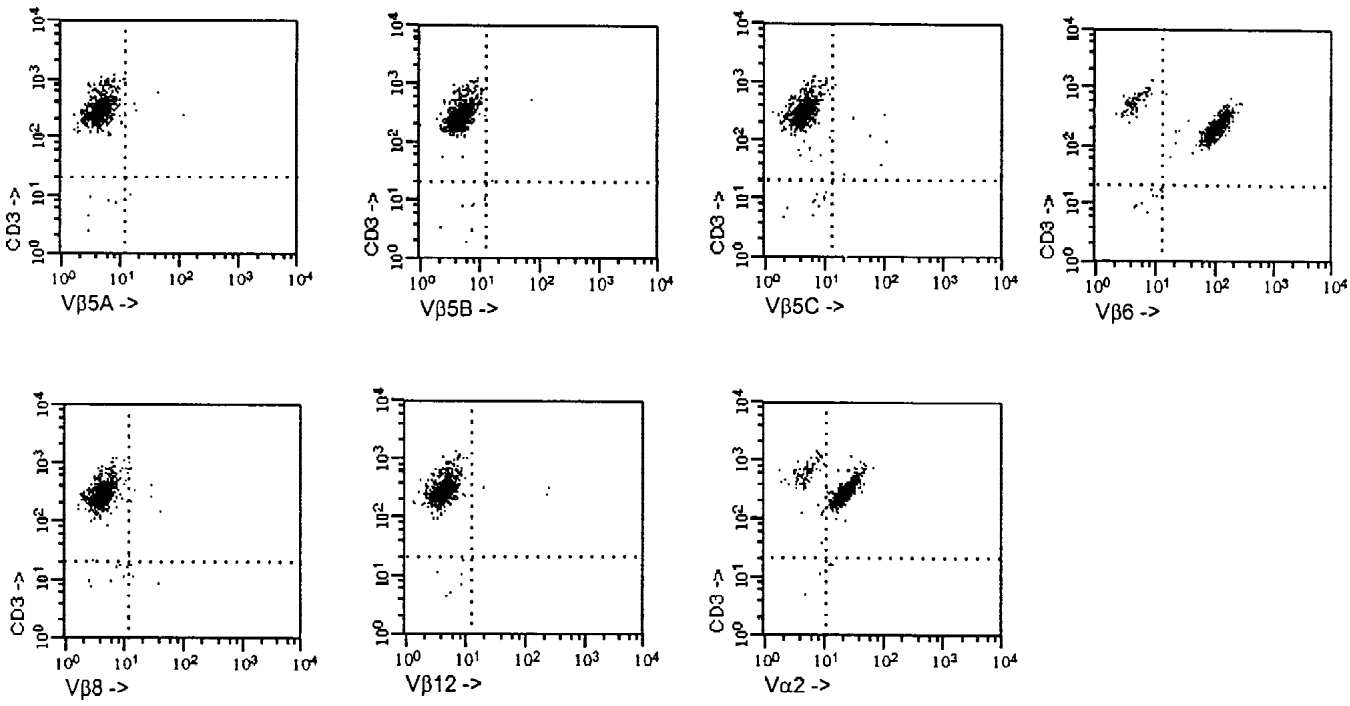

Fig 1. Flowcytometric expression of a T cell-line with preferential TCR family usage from rheumatoid synovium of a patient with early rheumatoid arthritis (RA 14). Dot plots representing T cell receptor family (fluorescein labeled) and CD3 (phycoerythrin labeled) expression are shown.

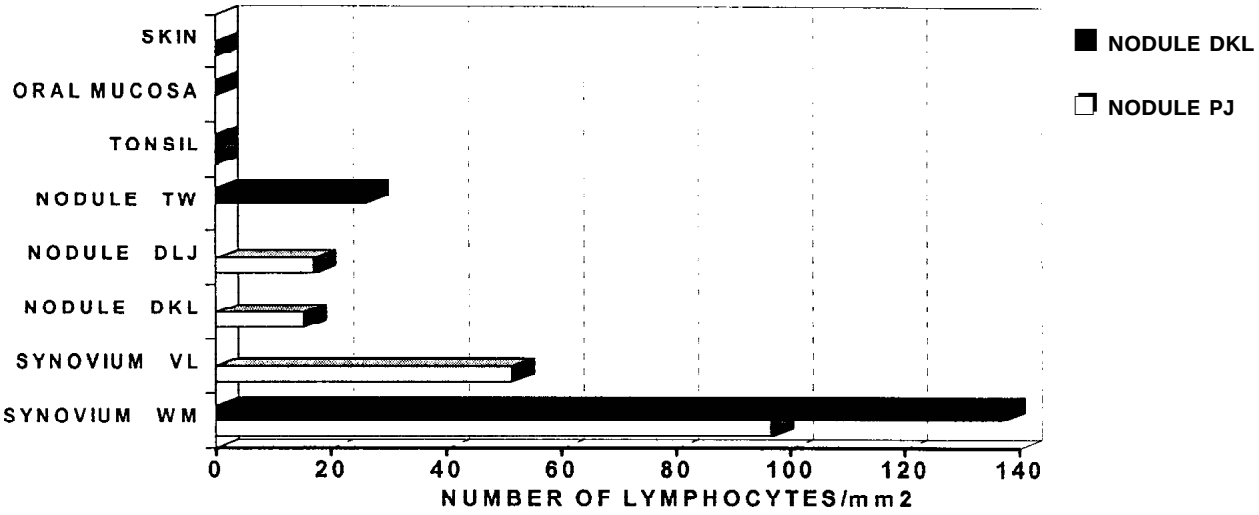

Fig 2. *In vitro* binding of T cell-lines from rheumatoid nodules from 2 RA patients (DKL and PJ). Frozen tissue sections (5 µm) from rheumatoid nodules, synovium, tonsil or skin were overlayed with 500,000 T cells labeled with an anti-CD45RO antibody and incubated for 30 minutes at 4°C. Non-adherent cells were then removed by washing in PBS. T cells bound to the substrate were visualised with the chromogen aminoethylcarbazole. Results are expressed as the number of adhering memory T cells/mm^2 tissue.

patient, indicating that the TCR selection is influenced by the intrinsic individual immune system of the host. The specificity of the bias in the TCR spectrum in subcutaneous nodules is supported by the overexpression of the same TCR families in different cell-lines from the same nodule, and even more interestingly, in different T cell-lines expanded from different nodules of the same RA patient. The restricted TCR family usage was striking, as one-third of the generated T cell-lines showed a > 30% overexpression of a given TCR family.

Subsequently, the TCR spectrum of synovial T cell-lines from 7 RA patients with long-standing disease and 8 RA patients with early disease was analyzed, using the same approach. In contrast with the results in long-standing RA, a high frequency of synovial T cell-lines with restricted TCR family usage was noted in early RA patients. Moreover, the TCR spectrum in these patients was skewed towards Vα2.

The observation that the TCR spectrum among IL-2 expanded synovial T cell-lines is significantly broader in long-standing disease may fit into the concept of epitope and antigen spreading during the evolution of the inflammatory process, as it has been described in experimental models of autoimmune disease (14-17). In previous reports on T cell clonality in RA synovial fluid, a similar oligoclonality in early but not late RA has been documented (18). In addition, in a limited set of early RA patients (N:3) a highly restricted Vα repertoire was noted in RA synovial tissue (19). Vα2 expansions in RA synovial fluid were also reported by Broker *et al.* (20). In another collaborative study, we analysed TCR expression in synovial membrane of early RA patients at 2 time-points by PCR-ELISA (21), and showed a reduction in the number of overrepresented V genes in the synovial biopsies over a period of 4 months (22). Together, these observations are best explained by a broadening of T cell recruitment and activation with time during the inflammatory process.

Since antigen-specific T lymphocytes are thought to play a pivotal role in the pathogenesis of rheumatoid arthritis, it can be postulated that these T cells recirculate between the different sites of tissue inflammation (mainly synovial membrane and rheumatoid nodules). The detection of identical T cell clones in different joints in one RA patient (23, 24) indeed supports this concept. There is as yet no such observation in synovial membrane and rheumatoid nodule in an individual patient. As an alternative approach to that question, we examined *in vitro* cross-adherence between rheumatoid synovium and nodules by an *in vitro* binding assay on frozen tissue sections. We observed preferential *in vitro* adherence of nodular T cell-lines to rheumatoid synovium and rheumatoid nodules, compared to control tissues (including inflamed tissues). This correlates with our earlier findings of: (i) similar expression of T lymphocyte homing molecules on expanded cell-lines from RA synovium and nodules; and (ii) similar expression of endothelial ligands in both types of tissue, which also supports a recirculation hypothesis between articular and extra-articular manifestations in RA (25).

The restriction of TCR family usage in expanded T cell-lines from rheumatoid nodules and early RA synovium, but not in longstanding RA or control tissues, constitutes the main finding of this study. The biological significance of this phenomenon and the role of these identified T cells in the pathogenesis of the disease remain to be resolved. The finding of similar TCR overexpression (in terms of TCR family usage) in cell-lines from different rheumatoid nodules obtained with time interval from single patients, and our previous observation that CDR3 sequences in these cell-lines are related, argue in favour of a specific significance (in terms of antigen-induced activation) of these cell-lines. One may consider that these cell-lines reflect a population of T cells with specific antigen recognition, which fulfill a specific immunomodulatory role in the pathogenesis of arthritis.

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