Clinical and laboratory study

T-CELL RECEPTOR GENE REARRANGEMENT STUDIES IN SÉZARY SYNDROME.

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ABSTRACT

Sézary syndrome (SS) is a cutaneous T-cell lymphoma characterized by pruritic erythroderma, generalized adenopathy and circulating Sézary cells in peripheral blood. The Sézary cells' percentage and morphology necessary for the diagnosis are still unknown; therefore, recently T-lymphocyte receptors gene rearrangement has been employed thus allowing to study the monoclonality of lymphoproliferative disorders. In order to verify the specificity and the diagnostic relevance of the above mentioned method, the DNA obtained from the peripheral blood of 17 subjects (10 affected by SS and 7 by inflammatory erythroderma) was analyzed by the Southern Blot Analysis (SBA). A monoclonal gene rearrangement of the TcR ß-chain was shown in 6 subjects affected by SS. In 2 patients the analysis was repeated during the therapy with recombinant interferon α-2a (rIFNα-2a), in one patient the clonal population had disappeared. In 4 patients with CSCs and germline pattern in the peripheral blood ("false negatives") gene rearrangement bands were obtained in skin and lymphnodes except for in one patient. Seven controls with inflammatory erythroderma presented a germline pattern.

These results suggest that the TcR gene rearrangement, although it is a useful diagnostic tool, must always be associated with clinical, histopathological and immunophenotypical data; Furthermore, this study highlights the need to make consecutive sampling, possibly with material from various organs (peripheral blood, lymphnodes, skin).

KEY WORDS

T-cell receptor, gene rearrangement, Southern blot analysis, cutaneous T-cell lymphoma

INTRODUCTION

Sézary syndrome (SS) is a cutaneous T cell lymphoma (CTCL) characterized by pruritic erythroderma, generalized adenopathy and circulating atypical lymphoid cells in peripheral blood (circulating Sézary cells, CSC) (1). There are different opinions regarding the percentage of CSC (2,3,4,5) and the morphological criteria (5,6) necessary to confirm the diagnosis: Cases of SS with moderately atypical nuclei of the circulating lymphocytes (7) and, on the contrary, reactive dermatitis or benign lymphoproliferative

Table I. Patients with Sézary syndrome (SS) and the stage of their disease at the time of investigation.

PATIENTS	AGE / SEX	CLINICAL FEATURE	STAGE
1	41/F	erythroderma	$T_4N_0M_0$
2	62/F	erythroderma	$T_4N_OM_1$
3	65/F	erythroderma	$T_4N_0M_1$
4	/F	plaques>10%	$T_2N_0M_0$
5	72/M	erythroderma	$T_4N_0M_1$
6	45/F	erythroderma	$T_4N_0M_1$
7	61/M	plaques>10%	$T_2N_OM_1$
8	57/M	plaques>10%	T_2N0M_0
9	63/M	erythroderma	$T_2N_0M_0$
10	43/M	erythroderma	$T_2N_1M_0$

disorders showing lymphoid Sézary-like cells in peripheral blood are known (2,5,8).

The evaluation of the peripheral T-lymphocyte -ß-chain receptor (TcR ß) gene rearrangement allows to evaluate the monoclonality of a lympho-proliferative infiltrate in skin (9,10,11), lymphnodes (12) and peripheral blood (6,9,13). In the majority of peripheral T-lymphocytes, the TcR is made of two proteic α/ß chains; only a small percentage expresses an alternative heterodimeric γ/δ chain (13,14,15). All the four chains are coded by genes with a configuration similar to immu-noglobulins (Ig); they are formed by the assembling of numerous VJC (α and γ) and VDJC (ß and δ) segments (Fig. 1). Such genic order defines the "germline" configuration. During the thymocyte differentiating process the VDJC segments are juxta-posed thus originating a "somatic-physiological rearrangement" specific for each T-cell (14). The clonal cells which originate from the T-lymphocyte proliferation have the same type of gene receptor rearrangement which is detected analyzing the DNA by Southern blot analysis (SBA).

In order to verify the SBA sensibility and the specificity as well as the diagnostic and prognostic importance of this method, we analyzed the DNA obtained from peripheral blood of 17 subjects, 10 of whom were affected by SS and 7 by inflammatory erythroderma.

In subjects affected by SS showing "false negative" results in molecular analysis of peripheral blood, in spite of the presence of a various percentage of CSC, the gene rearrangement was analyzed also in skin and lymphnodes. In two patients the band alterations of the TcR ß gene rearrangement in peripheral blood were analyzed during the maintenance phase of the parenteral therapy with rIFNα-2a.

MATERIALS AND METHODS

Subjects: We selected 10 subjects, 6 women and 4 men 41 to 72 years old, affected by SS who showed typical clinical manifestations (7 patients with erythroderma, 3 with widespread patches >10%), with the Karnofsky index >60% and life expectation >12 months. The clinical diagnosis was confirmed by histopathological and immunophenotypical examination (Table II). The staging of the disease was defined according to the TNM classification of the T-cell lymphoma (Bunn et al, 1979) (Table I) (16). Two patients (case 1 and 2) had long-lasting inveterate mycosis fungoides (MF). The molecular investigations were made on all the subjects prior to the beginning of the therapy and were repeated on two patients (case 3 and 10) during the maintenance phase of the parenteral therapy with an average dose of $9x10^6$ IU/day rIFNα-2a. Furthermore, in 4 patients with SS the molecular investigation was made in the skin (case 5-7-9) and in the lymphnode (case 6). The molecular study on peripheral blood of 7 patients affected by inflammatory erythroderma was also made.

Samples: Following centrifugation on Lymphoprep, the fraction of mononuclear cells of peripheral blood (in EDTA) was obtained.

DNA extraction: The samples were DNA extracted and purified utilizing the ONCOR DNA Extraction Kit.

The lymphnodes were incubated overnight at 60°C with the lysis buffer containing proteinase. Following incubation, the digested proteins were removed through double treatment with phenol and then with chloroform. The DNA precipitated, with cold ethanol and re-suspended in TRIS-EDTA (TE). 10 µg DNA of all the samples were digested with the restriction

Table II. The results of the immunological and molecular investigations.

PATIENTS	LEUKOCYTES c/µl	LYMPHOCYTES c/µl	SÉZARY CELLS (%)	CD4/CD8 RATIO	S.B.A. -TcR
1	3840	1100	10	1,4	G.L.
2	19100	8500	15	14,6	G.L.
3	6430	1800	10	5,2	R.
4	9040	2010	6	5,1	R.
5	9000	3100	10	9,9	G.L
6	16400	6230	5	30,3	R.
7	5580	2050	5	1,2	G.L.
8	8430	2400	6	3	R.
9	7540	2140	0	2,5	R.
10	5700	1800	5	1,5	R.

Legends:
G.L.= germline pattern
R= rearrangement

enzymes EcoRI, BamHI and Hind III. The DNA was submitted to electrophoresis on 0.7% agarose gel on Probe Tech 2 (ONCOR). At the same time, a sensitivity control (ONCOR), containing a mixture of 0.25 µg of EcoRI cut placenta DNA, 0.25 g of BamHI cut placenta DNA, and 0.25 µg of Hind III cut placenta DNA, was also submitted to electrophoresis. This DNA is mixed so that germline fragment bands from each enzyme are included. If these bands are visible, the test detects the equivalent of either: 1) a clonal population that contains one rearranged allele of - gene which is as small as 5% of the total sample population, or 2) a clonal population containing two rearranged alleles of the same gene as small as 2.5% of the total sample population. The darker the bands of the sensitivity control, the more sensitive the test. The lowest limit of detection represents a clonal population of 1% when both alleles of ß-gene are rearranged or of 2% when only one allele is rearranged.

Following depurination and denaturation, the separated fragments were vacuum transferred on nylon filters. After drying at 80°C in the oven, the filters were pre-hybridized.

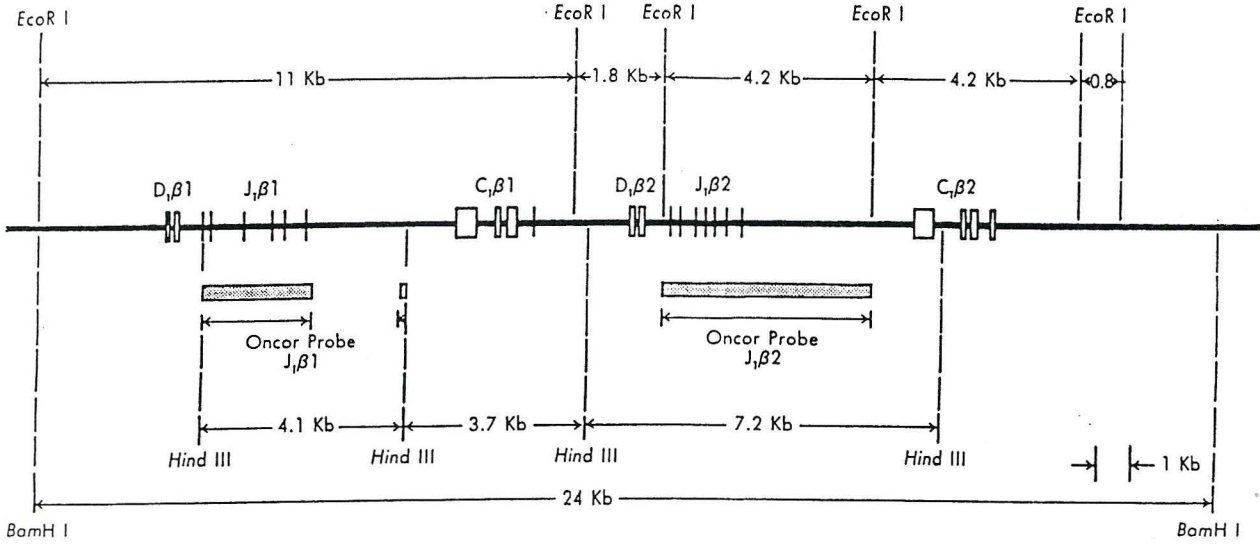

Fig. 1. Map of the gene codifying of the TcR -chain.

HYBRIDIZATION

The filters were hybridized with the biotynilated genomic probe (ONCOR $J\beta_1/J\beta_2$), complementary to the J region of the TcR α/ß chain. Following hybridization (18 hours at 50°C), the filters were washed with SSC 0.1/SDS 0,1%; then they were incubated with streptavidin and alkaline phosphatase, washed with SSC 1 and stained with a NBT chromogen and BCIP (Blue tetrazolium and 5-bromo-4-chloro-3-indolphosphate) at 37°C for 4 to 16 hrs. Following visualization of the bands, the filters were washed in distilled water, dried and kept in a dark place.

RESULTS

The results are summarized in Table I. The clonal rearrangement bands were observed in peripheral blood in 6 patients affected by SS (cases 3-4-6-8-9-10); in the other 4 patients (cases 1-2-5-7) the pattern resulted to be germline; after further investigations (cases 5-6-7-9), the TcR ß gene rearrangement bands were observed in skin (cases 7-9) and one lymphnode after histopathological examination revealed a pathologic result (case 6). In two patients (cases 3-7) the molecular investigation was repeated during the therapy with rIFNα-2a: in one patient (case 3) the CSC and the clonal rearrangement band of the TcR ß in peripheral blood disappeared. Seven patients with inflammatory erythroderma (one showed 15% of Sézary-like lymphoid cells in circulation) showed a DNA germline pattern in peripheral blood. Sensitivity control was visible in all analyses of rearrangements performed, indicating that the tests were able to detect a clonal population containing one rearranged allele as small as at least 5% of the total sample population or two rearranged alleles as small as 2.5% of the total sample population.

In Fig. 2 the DNA rearrangement bands obtained by SBA of patients 3-4-10 are shown. The DNA, digested by the enzymes EcoRI, BamHI and Hind III, was hybridized with the $J\beta_1/J\beta_2$ genomic probe. The rearrangement bands are marked with arrows.

DISCUSSION

The number and the morphology of CSC necessary for the diagnosis of SS are still unknown.

Recent investigations of peripheral T-cell ß-chain

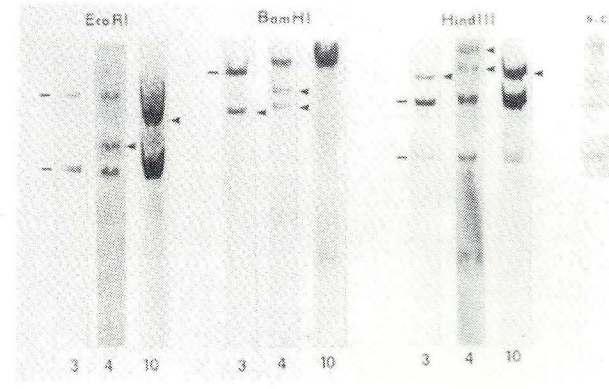

Fig. 2. DNA Southern Blot Analysis of patients 3-4-10 in peripheral blood. The digested DNA with the enzymes EcoRI, BamHI and Hind III was hybridized with the genomic $J\beta_1/J\beta_2$ probe. The bars indicate the germline bands; the arrows indicate the rearrangement bands. S.C. indicates the sensitivity control.

receptor (TcRß) gene rearrangement, analyzed by the SBA, to evaluate the clonality of a lympho-proliferative infiltrate in skin (9,10,11), lymphnodes (12) and peripheral blood (6,9,13) resulted in a high sensitivity (80-100%) and specificity (>90%) (6,15,17,18,19).

The DNA obtained from peripheral blood of 17 patients, 10 affected by SS and 7 by inflammatory erythroderma, was analyzed; the results of the molecular investigations were compared with the morphological and immunological data (Table II): the patients (case 3-4-6-8-9-10) showed a TcR ß clonal rearrangement in peripheral blood samples, in absence of direct correlation with the number of CSC and the extension of the skin lesions. Weiss (6) studied the TcR clonal rearrangement in peripheral blood of 26 patients affected by MF/SS: 3 patients with SS presented TcR ß gene rearrangement in correlation with the CSC presence; 2 patients showed a germline pattern with 3%-4% CSC. Witthaker et al. (11) showed in 8 patients out of 14 a clonal TcR rearrangement in peripheral blood samples, while the remaining 6 patients formed a germline pattern with a variable CSC percentage (0-5%). According to these results, it is possible to find a TcRß germline pattern in peripheral blood in the presence of a small number of CSC. On the contrary, in one patient (case 9) TcR ß rearrangement bands without morphological CSC occurred. SS variants with a modest atypia of CSC nuclei (7) have also been published.

Four patients (case 1-2-5-7) presented a germline

pattern, in peripheral blood (case 1-2) and in the skin (case 5), in spite of the high percentage of CSC. Bendelac (20) showed a case of acute non epidermotropic T-cell lymphoma with 67% CSC in the absence of the TcR ß gene rearrangement but with rearrangement bands in the affected skin. The observation may be explained in different ways: the rearrangement band might have co-migrated with the germline band or a deletion of chromosomic segments containing TcR genes occurred or those atypical cells were activated polyclonal T-lymphocytes with a reactive value; furthermore, the use of the biotynilated genomic probe, complementary at the J region of the α/ß TcR ß chain, might not recognize the rearrangement of the γ/δ chain (2,5,6,8,12,13).

Case 6 shows similar monoclonal rearrangement bands in peripheral blood and in an affected lymphnode thus showing the monoclonality of the neoplastic infiltrate. In fact, the monoclonality of an infiltrate doesn't necessarily prove its malignancy but may indicate an abnormal cellular proliferation with possible progression to malignancy (9). The diseases with a benign course (acute lichenoid pityriasis, lymphomatoid papulosis) present clonal proliferation of reactive T-lymphocytes (15). In our case, the presence of the same clonal population infiltrating the lymphonode and peripheral blood determined the diagnosis, although numerous reactive lymphocytes overlapped the infiltrate (12).

In case 7 the rearrangement bands were shown in skin and not in peripheral blood, even if morphologically 5% CSC were present. A similar observation has already been described in literature (11). The authors hypothesize that in CTCL the circulating polyclonal reactive lymphoid cells with a DNA germline pattern are different from the clonal neoplastic cells which infiltrate lymphnodes and skin. A clinical and biological explanation is at present not available (6).

During the maintenance phase of the parenteral therapy with rIFNα-2a at an average dose of $9x10^6$ IU/day in 2 patients, one (case 3) showed a partial clinical remission with the disappearance of CSC, the clonal TcR ß rearrangement bands in peripheral blood and an improvement of the CD4/CD8 ratio, while in the other (case 10), the immunological and molecular pattern was not changed. In literature one SS case with TcR gene rearrangement bands in peripheral blood has been reported, who after combined therapy with rIFNα-2a and extracorporeal photochemotherapy displayed a germline pattern (21). These results indicate that the TcR ß clonal rearrangement studies by serial withdrawals of peripheral blood allow to monitor eventual molecular variations, possibly correlated with the clinical evolution of the disease. All the 7 patients with inflammatory erythrodermia showed a germline pattern, although one of them had 15% Sézary-like cells in circulation. Bakels et al. (19) showed an inflammatory erythroderma with clonal rearrangement of the TcR gene in peripheral blood.

These data appear to be consistent with those of other authors (6,9,13,19) in which the results of the TcR ß clonal rearrangement studies in peripheral blood were extremely sensitive. The use of the Polymerase Chain Reaction (PCR), the Denaturing Gradient Gel electrophoresis (DGGE) (22,23,24,25,26) and the withdrawals performed at different times (peripheral blood, skin, lymphnodes) may increase the sensitivity of the molecular investigations and thus strengthen the diagnosis.

CONCLUSIONS

Molecular biology makes possible a more accurate diagnostic evaluation, as well as the monitoring of the therapeutical effects and a better understanding of the etiopathological events of the lymphoproliferative diseases. The results have to be compared however with the clinical, histological and immunopathological investigations, therefore they represent a complementary diagnostic tool.

ABBREVIATIONS' LIST

SS	=	Sézary Syndrome
MF	=	Mycosis Fungoides
TcR	=	T-cell Receptor
rIFN- α	=	recombinant Interferon alfa
CSC	=	Circulating Sézary Cells
CTCL	=	Cutaneous T Cell Lymphoma
DNA	=	Deoxyribonucleic Acid
SBA	=	Southern Blot Analysis
EDTA	=	Ethylenediaminetetraacetic acid
TE	=	Tris-EDTA
SSC/SDS	=	Saline-sodium Citrate/ Sodium Docecyl sulfate
NBT	=	Nitroblue Tetrazolium
BCIP	=	5-bromo-4-chloro-3-indolphosphate
PCR	=	Polymerase Chain Reaction
DGGE	=	Denaturing Gradient Gel Electrophoresis

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