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Infrequent Fas Mutations but No Bax or p53 Mutations in Early Mycosis Fungoides: A Possible Mechanism for the Accumulation of Malignant T Lymphocytes in the Skin

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Mycosis fungoides (MF) is the most frequent manifestation of cutaneous T cell lymphoma but its cause and pathophysiology remain unclear. Because progression of lesions is characteristically slow, we hypothesized that mycosis fungoides originates from an accumulation of lymphocytes due to defective apoptosis of skin homing T lymphocytes. In this study, we investigate possible alterations of three molecules regulating apoptosis, i.e., Fas antigen, Bax, and p53, at the genomic level in skin lesions from 44 patients with MF, as Fas mediates one of two major pathways for apoptosis of activated T cells. Fas mutations were found in six patients using a polymerase chain reaction and single-strand conformational polymorphism method followed by cloning and sequencing of abnormal polymerase chain reaction products. The mutations predict for defective transmission of the death signal in three cases. Immunohistochemistry demonstrated the lack of Fas protein expression on dermal lymphocytes in one case with Fas gene mutation predicting for a truncated death domain, whereas Fas protein was expressed by dermal lymphocytes in the other investigated cases. By contrast, no mutations of Bax or p53 were found, whereas immunohistochemistry demonstrated increased p53 expression in the nucleus of basal keratinocytes above the neoplastic infiltrate in some MF cases. These results support the hypothesis that Fas defects may play a role in the pathogenesis of MF. ***Key words: apoptosis/ cutaneous T cell lymphoma/ physiopathology. J Invest Dermatol 118:949–956, 2002***

Mycosis fungoides (MF) is the most frequent primary cutaneous T cell lymphoma characterized by erythematous patches evolving to infiltrated plaques, nodules and less frequently tumors and erythroderma. The main histologic feature of MF is a superficial dermal, often epidermotropic, infiltrate composed of $CD4^+$ helper T lymphocytes expressing cutaneous lymphocyte antigen CLA (Picker *et al*, 1990). The pathogenesis of MF is poorly understood as viral (mainly EBV) and retroviral theories have not been substantiated to date (Hall *et al*, 1991; Pancake *et al*, 1995; Angel *et al*, 1996; Lessin *et al*, 1996; Jumbou *et al*, 1997; Wood *et al*, 1997), whereas chronic antigenic stimulation remains possible (Wood, 1995). Molecular and genetic studies focused mainly on p53, bcl-3, NFKB2/lyt-10, and Tal-1 are sparse in MF and have not demonstrated consistent genetic alterations (Beylot-Barry *et al*, 1995; Garatti *et al*, 1995; Lauritzen *et al*, 1995; Li *et al*, 1998), especially in the early stages. In particular, mutations of tumor suppressor gene p53 have been detected in only one case of plaque stage MF (van Haselen *et al*, 1997), whereas it seems more frequent in advanced, tumor cases but without high specificity (McGregor *et al*, 1999). Recent studies have shown hypermethylation and allelic loss, but no point mutation of the $p16^{INKa}$ gene in MF (Navas *et al*, 2000), and loss of heterozygosity on 10q with possible homozygous deletion of the tumor suppressor gene PTEN, mainly in advanced cases of MF (Scarisbrick *et al*, 2000).

An alternative hypothesis could consider early MF to develop from an accumulation of CLA^+ T lymphocytes rather than a neoplastic disease. Assessment of apoptosis in lymphoid cells of MF lesions had been done in only a small number of cases using the TUNEL method. The results were equivocal and no definite conclusion could be made (Kikuchi and Nishikawa, 1997). An initial accumulation of T lymphocytes would explain the usually protracted indolent course and the limited atypical features observed in early MF. According to this hypothesis, the initial event might be related to defective apoptosis occurring in a subset of activated T cells expressing CLA. Alterations of four major regulators of apoptosis, p53, Fas, bax, and bcl-2, have been reported in some hematologic malignancies. Specifically, mutations of Fas, one of the main regulators of postactivation apoptosis (Nagata, 1997), are present in non-Hodgkin's lymphomas (Gronbaek *et al*, 1998), HTLV-1-induced acute T cell leukemia of adults (Tamiya *et al*, 1998), acute lymphoblastic leukemia of childhood (Beltinger *et al*, 1998), and multiple myeloma (Landowski *et al*, 1997), whereas mutations of the pro-apoptotic gene bax with potential loss of function have been reported in non-Hodgkin's lymphomas (Meijerink *et al*, 1995; Brimmell *et al*, 1998; Meijerink *et al*, 1998; Peng *et al*, 1998; Gutierrez *et al*, 1999; Gaidano *et al*, 2000; Inoue *et al*, 2000; Miyauchi *et al*, 2000). Bax expression appears

Manuscript received September 25, 2000; revised January 9, 2002; accepted for publication January 21, 2002.

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Abbrevations: ALPS, autoimmune lympho–proliferative syndrome; CTCL, cutaneous T cell lymphoma; MF, mycosis fungoides.

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Table I. Primers used for PCR-SSCP analysis of Fas

Primers	Sequence	Size of the product
1-F	5′-GGAAGCTCTTTCACTTCGGA-3′	116 bp
1-R	5′CTAAGACGGGGTAAGCCTCC-3′	
2-F	5′-GTGGGTTACACTTCTTTACCACGTTGC-3′	216 bp
2-R	5′-CATGATTACTATGTGCTACTCCTAACTG-3′	
3-F	5′-CCGTGTCCTGTTCAAACACTT-3′	220 bp
3-R	5′-CCAAGATTGGCCTCTTTCAA-3′	
4-R	5′-CTGCTTATAATTAGCCGCGA-3′	228 bp
4-F	5′-ACTTCCCTAGGAAAATGATAACTCT-3′	
5-F	5′-TGCCAGGCTTTTGAATTTCT-3′	161 bp
5-R	5′-TCACATCTTTCCCTGGGGTT-3′	
6-F	5′-CAATCACTCTTGATTAGTAGAAAGTCC-3′	210 bp
6-R	5′-TTGTGAACTACTTCCCCCAAG-3′	
7-F	5′-CATGCATTCTACAAGGCTGAG-3′	178 bp
7-R	5′-TTTTCTTTTCAAGGAAAGCTGAT-3′	
8-F	5′-TTTATTTGTCTTTCTCTGCTTCCA-3′	123 bp
8-R	5′-GGCCTATTACTCTAAAGGATGCC-3′	
9–1F	5′-TGGGAATTTCATTTAGAAAAACAA-3′	261 bp
9–1R	5′-TGTCATACGCTTCTTTCTTTCC-3′	
9–2F	5′-CAATGTCCAAGACACAGCAGA-3′	246 bp
9–2R	5′-TACTCAGAACTGAATTTGTTGTT-3′	

Table II. Primers used for PCR-SSCP analysis of Bax

Primer	Sequence	Size of the product
1F	5′-CGTTCAGCGGGGCTCTCA-3′	207 bp
1R	5′-CAGGCCGGTAGGAAGGAT-3′	
2–3F	5′-CCCCTAGAACCCAAGAGTC-3′	400 bp
2–3R	5′-GGCTGAGAGTCCTGTGTCC-3′	
4F	5′-TCTCCTGCAGGATGATTGC-3′	209 bp
4R	5′-TCCCCAGGTCCTCACAGAT-3′	
5F	5′-CAGGCAGTGGGGACAAGGTT-3′	192 bp
5R	5′GCGGTGGTGGGGGTGAGGAG-3′	
6F	5′-CCCCTGGCCGAGTCACTGAA-3′	237 bp
6R	5′-AATGCCCATGTCCCCCAATC-3′	

Table III. Primers used for PCR-SSCP analysis of p53

Primer	Sequence	Size of the product
5F	5′-GCCGTGTTCCAGTTGCTTTATC-3′	186 bp
5R	5′-GTAGATGGCCATGGCGCGGACG-3′	
5′F	5′-GTGGATTCCACACCCCCGCCCGG-3′	192 bp
5′R	5′-TCAGTGAGGAATCAGAGGCC-3′	
6F	5′-CTGGAGAGACGACAGGGCTG-3′	236 bp
6R	5′-GCCACTGACAACCACCCTTA-3′	
7F	5′-CCTCATCTTGGGCCTGTGTT-3′	202 bp
7R	5′-TCAGCGGCAAGCAGAGGCTG-3′	
8–9F	5′-AGGACCTGATTTCCTTACTTG-3′	390 bp
8–9R	5′-CTTTCCACTTGATAAGAGGTC-3′	

decreased in low-grade lymphoid malignancies compared with more aggressive diseases, which supports impaired bax-mediated apoptosis as a basic mechanism in low-grade lymphomas such as MF (Soini *et al*, 1998; Wheaton *et al*, 1998; Camillieri-Broet *et al*, 2000). There are similarities in the slow clinical evolution and outcome of MF and follicular nodal lymphoma, a condition clearly related to overexpression of antiapoptotic bcl-2 (Korsmeyer, 1999; Stamatopoulos *et al*, 2000). Finally, a dominant autosomal autoimmune lymphoproliferative syndrome (ALPS), characterized by prominent accumulation of lymphocytes in lymphoid organs, has been recently described and attributed in most cases to dominant negative germline mutations in the Fas gene (Fisher *et al*, 1995; Rieux-Laucat *et al*, 1995; Drappa *et al*, 1996; Bettinardi *et al*, 1997; Dianzani *et al*, 1997; Sneller *et al*, 1997).

Our hypothesis has led us to search for alterations in genes regulating apoptosis of T cells, namely fas, Bax, and p53 in MF. We examined the nine exons of the translated part of the Fas gene, the six exons of the coding part of the bax gene, and exons 5–9 of the p53 gene by exon-specific polymerase chain reaction (PCR) and single-strand conformational polymorphism (SSCP) analysis, along with Fas, Bax, and p53 expression by immunohistochemistry in cutaneous lesions of 44 MF patients.

MATERIAL AND METHODS

Tissues and patients Forty-four cutaneous biopsies from 44 patients with MF were selected randomly on the basis of available paraffin blocks and frozen tissues. In all cases, the diagnosis of MF was confirmed by clinical, histologic, and, in all but three cases, immunophenotypic criteria. Biopsies were performed on patches (24 cases), plaques (17 cases), and tumors (three cases). Neoplastic cells with convoluted nuclei resembling Sezary cells were present with some degree of epidermotropism in each case. Tissues used for DNA extraction were either snap-frozen or paraffin-embedded. Paraffin-embedded biopsies from eight patients with lichen planus were used as controls for the molecular study of Fas, Bax, and p53, and for Bax and Fas immunohistochemistry. Additional frozen samples from 10 patients with eczema were used as a negative control group for p53 immunostaining.

Genomic DNA extraction Because the only available tissues for some patients were formalin-fixed, paraffin-embedded samples, we used genomic DNA instead of cDNA for all samples. To limit the background of normal keratinocyte DNA, we performed microdissection in 29 of 44 MF samples prior to DNA extraction, carefully removing by hand with a 30 gauge needle the upper part of the dermal infiltrate under microscopic control on 25 μm thick tissue sections. This procedure eliminated most of the epidermal background and improved detection of the T cell clone probably by an increase in DNA from neoplastic cells (data not shown, manuscript submitted). Genomic DNA was extracted from both snap-frozen and paraffin-embedded tissues. Paraffin slides were dewaxed and submitted to overnight digestion with 0.1 mg proteinase K at 37°C. After desalting with 0.5 vol of NaCl 5 M and centrifugation, genomic DNA in clear supernatant was precipated with two volumes absolute ethanol. Pelleted DNA was washed with 70% ethanol, dried, dissolved in 50 μl Tris 10 mM, pH 7.6, EDTA 1 mM, pH 8 and stored at 4°C until further use.

PCR amplification Primer pairs were designed to amplify the coding region of the Fas gene (i.e., exons 1–9), according to previous reports (Beltinger *et al*, 1998) or with the help of Primer 3 software (Whitehead Institute for Biomedical Research of the Massachussets Institute of Technology, Cambridge, MA, web site: http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The entire coding region of the bax gene (i.e., exons 1–6) and exons 5–9 of the p53 gene were amplified according to previous reports. Because of the length of exon 5 of p53, it was amplified with two different sets of primers leading to two overlapping amplicons. Detailed sequences of primers and the size of the expected products are showed in **Tables I**, **II** and **III**. Radioactive PCR was performed on a Perkin-Elmer-Cetus N801-0150 DNA Thermal Cycler, with 100 ng of genomic DNA, 250–50 nmol per liter of each primer, 2.5 μCi of ^{32}PdCTP and Ready-to-Go PCR beads (Pharmacia Biotech, Piscataway, NJ) 200 μmol per liter each of dATP, dCTP, dTTP, and dGTP; 50 mmol KCl per liter; 1.5 mmol $MgCl_2$ per liter; 10 mmol Tris-Hcl pH 9.0 per liter; 1.5 units of Taq DNA polymerase with a final volume of 25 μl. The samples were denatured at 95°C for 5 min then amplified for 35 cycles consisting of 1 min at 95°C, 1 min at a temperature range of 58–65°C, depending on the primer pair and 2 min at 72°C, followed by a final extension segment of 10 min at 72°C. Quality of the PCR was controlled by migration of a sample amplified without radioactive nucleotide in a 2% agarose gel.

Table IV. Summary of clinical, histologic, and molecular data of the six MF patients with Fas gene alterations

Patient	Clinical data	Histopathology	Sequence changes[a]	Amino acid changes
1	Patches and plaques	Upper dermal infiltrate with epidermotropism	C313T	THR 40 ILE
2	Plaques	Upper dermal infiltrate	G448A	CYS 85 TYR
3	Patches and plaques	Upper dermal infiltrate with epidermotropism	A535G	GLU 114 GLY
4	Rapidly progressing tumors	Dense overall dermal infiltrate	Insertion G 765 stop at codon 211	Frameshift with premature codon
5	Patches	Upper dermal infiltrate	Deletion G 846 stop at codon 222	Frameshift with premature codon
6	Plaques	Upper dermal infiltrate with epidermotropism	A849C	THR 219 PRO

[a]Expressed in cDNA nucleotide number according to Itoh *et al* (1991).

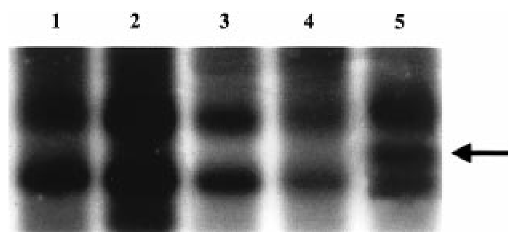

Figure 1. PCR-SSCP analysis of Fas antigen in MF patients skin samples. ^{32}P-labeled PCR products from exon 8 are separated on a 7% nondenaturing polyacrylamide gel. *Lanes 1–4* show a normal pattern of two bands in four patients. The *arrow* in *lane 5* indicates an abnormal band excised for cloning and sequence analysis detected in the lesion of patient 5.

Single-strand conformation polymorphism analysis Five microliters of radiolabeled amplified DNA was diluted in 5 μl of loading buffer (95% formamide, 5% EDTA 0.5 M pH 8, 0.025% bromophenol blue, 0.025 xylene cyanol blue), denatured at 95°C for 10 min, immediately cooled on ice for 5 min and loaded onto a nondenaturing polyacrylamide gel. Gels consisted of 0.5 × glycerol tolerant buffer (Amersham Life Science, Arlington Heights, IL), 5% glycerol and acrylamide (29:1 bi) 4–6.5% according to the size of the PCR product. Electrophoresis was carried out with 0.6 watts for 18–20 h at room temperature. Autoradiography was performed at –80°C overnight or longer if necessary. When band shifts were observed, a second round of radioactive PCR-SSCP was carried out for confirmation. Additional bands were then excised from the gel and DNA was extracted and purified using the QUIAEX II gel extraction kit (Quiagen, Santa Clara, CA). Extracted DNA was then re-amplified for 35 cycles with the same primer pair.

Cloning and sequencing analysis PCR products from the previous step were cloned using the TOPO-TA cloning kit (Invitrogen, La Jolla, CA). The recombinant plasmids were extracted with the QUIA Miniprep Spin kit (Quiagen) and sequenced with an automated Perkin-Elmer ABI Prism 377 XL sequencer using the Prism DNA Sequencing Analysis version 3.0 software and dye terminators. Three to five clones for each shifted band were selected for sequencing. The obtained sequences were analyzed and aligned using the Blast software from the National Cancer Institute, National Institute for Health, Bethesda, MD (website: http://www.ncbi.nlm.nih.gov/BLAST/).

Immunohistochemistry Five micrometer thick sections were cut from paraffin-embedded MF and lichen planus (Bax, Fas) or frozen tissues of MF and eczema (p53). For Bax and Fas immunostaining, the slides were baked at 60°C for 45 min, dewaxed and submitted to an antigen retrieval procedure in a 10 mmol sodium monochlorydrate per liter bath heated to 95°C for 12 min and then cooled for at least 30 min. Fas and Bax immunohistochemistry were carried out with a murine monoclonal anti-Fas antibody (Clone APO-1; Dako, Carpenteria, CA) diluted at 1:100 and a murine polyclonal anti-Bax antibody (Dako) diluted at 1:100, using the tyramine-amplified detection CSA kit (Dako) in both cases. Slides were counterstained with hematoxylin, dehydrated with ethanol, washed in xylene, and mounted with covering glasses. p53 immunostaining was performed on OCT embedded frozen tissue sections from MF and eczema with three different primary antibodies: two monoclonal antibodies (DO7 (Novocastra Laboratories, Newcastle upon Tyne, U.K.) and 1801 (Oncogene Science)) and one polyclonal antibody (CM1, Novocastra Laboratories, U.K.). These three antibodies recognize both wild-type and mutated versions of the protein. They were used at a 1/50 dilution in PBS/0.5% BSA and revealed with a streptavidin-biotin-peroxidase method (kit Immunstain, DPC). A Xeroderma Pigmentosum-induced epidermoid carcinoma with known mutation-induced p53 overexpression was used as a positive control. In all cases, negative controls included slides where the primary antibody was replaced by its diluent.

No loss of heterozygosity (LOH) assay was performed due to the unavailability of normal tissues for most patients and because of the heterogeneity of the dermal infiltrate in which neoplastic cells were often less numerous than normal or reactive cells.

RESULTS

Molecular analysis All specimens provided adequate genomic DNA for PCR. In Fas gene analysis, two rounds of radioactive PCR-SSCP analysis confirmed mobility shifts of exons 2, 3, 4, 7, and 8 in seven patients (**Fig 1**). No patient with lichen planus showed any mobility shifts with the nine primer pairs. The results of the sequencing procedures were considered valid only when at least two clones displayed the same result, and are summarized in **Table IV**. Sequence analysis revealed point mutations of the Fas gene coding sequence in six patients. Patient 1 displayed a C to T transition at bp 313 (exon 2) with subsequent substitution of THR to ILE at amino acid 40 (CDR1) (**Fig 2*a***). Patient 2 had a G to A transition at bp 448 (exon 3) with resulting substitution of CYS by TYR at amino acid 85 (junction CDR1-CDR2) (**Fig 2*b***). Patient 3 showed an A to G transition at bp 535 (exon 4), leading to a substitution of GLU to GLY at amino acid 114 (CDR2). In patient 4, an insertion of a G at bp 765 (exon 7) resulted in a frame shift and appearance of a premature stop codon at amino acid 211 with a truncated molecule lacking the death domain. Patient 5 had a microdeletion of a G at bp 846 (exon 8) resulting in a frame shift with premature stop codon at amino acid 222 with a predicted molecule lacking the death domain. The sample from patient 6 displayed an A to C transversion at bp 849 (exon 8) with a replacement of THR by PRO at amino acid 219. Patient 7 showed a mobility shift in the exon 3 corresponding to heterozygosity for the previously described polymorphism at bp 416 (A to G) (Fiucci and Ruberti, 1994).

PCR-SSCP analysis on both bax and p53 genes was carried out on 41 samples from MF and eight biopsies from lichen planus. The migration pattern of the six coding exons of bax gene displayed no abnormal band in any of the samples. An additional band was present in one MF sample only within exon 5 of the p53 gene, confirmed by a second round of PCR-SSCP. Cloning and

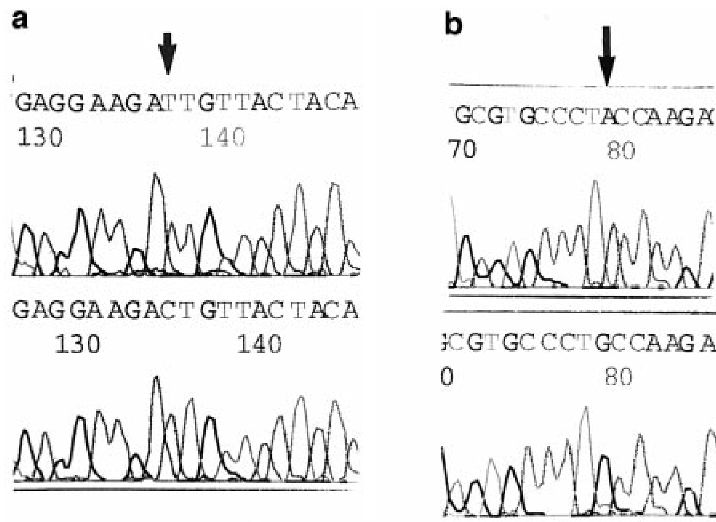

Figure 2. Sequence analysis of exon 2 (*a*) and 3 (*b*) -specific PCR product extracted from the additional band in SSCP gel from patients 1 and 2, respectively: point mutation (*arrows*) in position 313 with C to T transition (*a*) and 448 with G to A transition (*b*); normal sequences are shown below.

sequence analysis revealed a C to T transition at bp 596 of cDNA without any effect on the primary sequence of the protein and probably corresponding to a genetic polymorphism.

Immunohistochemistry Immunohistochemical analysis of Fas was performed in 19 of 44 MF and in five lichen planus cases. All MF cases displayed moderate to strong staining of basal, and to a lesser extent, of suprabasal layer keratinocytes in the epidermis and consistently strong staining of endothelial cells and sweat glands. Lymphocytes of the dermal infiltrate were faintly to moderately stained in all cases but one (including two patients with missense mutations of the Fas gene). Indeed, the patient displaying a mutation in exon 8 and subsequent frame shift with predicted disappearance of the death domain showed no staining of atypical lymphocytes, whereas the epidermis displayed the usual pattern (**Fig 3*a***). In most stained cells, a granular intracytoplasmic pattern associated with a membrane stain was observed. All lichen planus cases displayed stronger staining of the epidermis and the dermal inflammatory infiltrate than MF patients (**Fig 3*b***).

Bax expression was studied in 13 MF and eight lichen planus cases. In all cases, clear cytoplasmic staining of the dermal lymphoid cells was obtained with a similar intensity in MF and lichen planus (**Fig 4**). Immunostaining for p53 was carried out on 12 cryostat sectioned biopsies of MF and 10 of eczema with each of three antibodies. No staining was obtained in epidermis or in dermal infiltrate for eczema samples. Conversely, two cases of MF showed nuclear staining of some dermal lymphoid cells with a least two antibodies; some of the stained cells appearing as cytologicaly atypical (**Fig 5*a***). Furthermore, five of 12 MF patients (three patch-stage and two plaque-stage patients) consistently displayed nuclear staining of basal keratinocytes above the dermal infiltrate with at least one antibody, but in none of these five cases was there any staining of dermal neoplastic lymphocytes (**Fig 5*b*, *c***). Negative and positive controls displayed the expected results.

DISCUSSION

In this paper, we describe the presence of six point mutations of the coding sequence of the Fas gene in six of 44 patients with MF investigated (13%). The expression of Fas protein in lymphoid cells of the dermal infiltrate was absent in one case with a frame shift mutation, predicting for the disappearance of the death domain of the mutated allele. The low percentage of Fas mutations detected in MF must be interpreted in the context of limitations of the PCR-SSCP method used when genomic DNA is derived from a heterogeneous tissue containing both tumor and numerous reactive cells (Sheffield *et al*, 1993; Thor Straten *et al*, 1998); the percentage of tumor cells may be below the threshold of detection of PCR-SSCP. The functional significance of the identified mutations of Fas in these cases of MF is variable. The two cases with frameshift and premature stop codons, theoretically resulting in the disappearance of the death domain, are likely to have important functional consequences as this part of the molecule is crucial for transmission of the death signal (Cascino *et al*, 1996). A dominant negative effect of heterozygous death domain alterations, perhaps linked to the trimeric nature of the membrane receptor, is very likely in ALPS (Takahashi *et al*, 1994) and is suspected in plasma cell myeloma (Landowski *et al*, 1997) and non-Hodgkin lymphoma (Gronbaek *et al*, 1998). The lack of expression of Fas in the dermal lymphoid cells in one of the two cases with the possible disappearance of the death domain also supports the hypothesis of significant impairment of the Fas function, the truncation of the molecule possibly impairing formation of the membrane trimer. The significance of the missense mutations is less clear as they do not seem to be associated with loss of expression of the protein; however, one of them (G448A) leads to a nonconservative change of amino acid 85, very close to the two amino acids crucial for efficient binding of Fas-ligand (Starling *et al*, 1997), and may be functionally important. The significance of the three remaining missense mutations is unknown but it must be emphasized that two of them are nonconservative.

No relationship between the clinical or histologic pattern of lesions and the profile of mutations could be established, given the small number of cases with identified mutations. Most patients were of the usual type with chronic, patch, and plaque-type lesions, although one patient with a frame shift mutation had rapidly progressing tumors with a heavy dermal infiltrate, although without large cells. This presentation does not fit the model of slow lymphocyte accumulation, but subsequent mutations involving key genes may have occurred.

We hypothesize that defective apoptosis of chronically antigen-stimulated T cells is involved in some cases of MF through alterations of regulators of this apoptotic process, like the Fas-Fas Ligand system or the proapoptotic molecules Bax or p53, a theory of interest in CTCL in general (Meech *et al*, 2001). This hypothesis is based upon the following clinical and experimental observations. (1) The usual indolent course of the disease, consistent with an accumulation rather than a proliferation of lymphocytes, similar to follicular lymphomas where there is over-expression of apoptosis-inhibiting gene bcl-2 (Korsmeyer, 1999). (2) A hypothesis considering an etiologic relationship between chronic T cell activation in the skin and MF (Wood, 1995). (3) It has been recently reported that the Fas/Fas ligand system is likely to play a crucial role in the downregulation of T cell activation in contact dermatitis (Orteu *et al*, 1998). (4) Ultraviolet light therapy, a common treatment in the first stages of MF, seems to act by

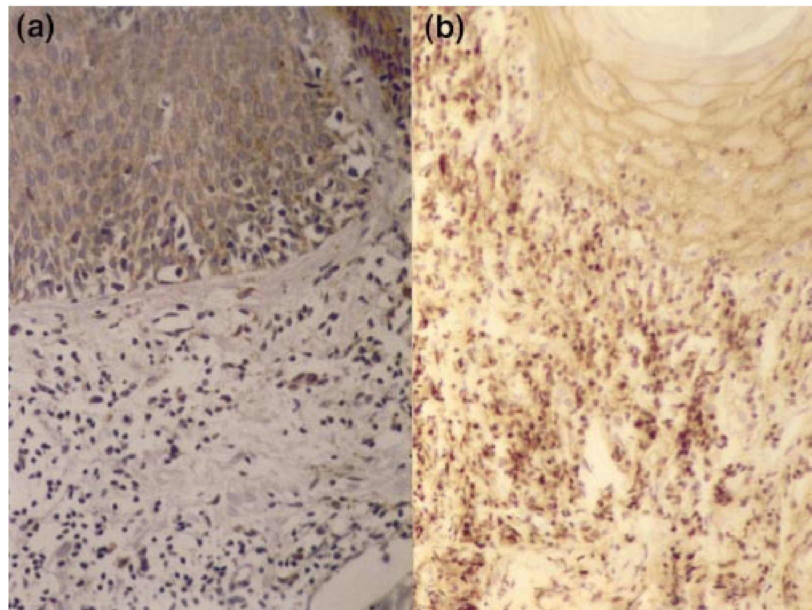

Figure 3. Immunohistochemical staining with anti-Fas antibody. (*a*) MF lesion of patient 5 with mutation and frameshift in exon 8 and subsequent loss of death domain in Fas antigen reveals staining of keratinocytes but not of malignant lymphocytes in the epidermis and dermis; (*b*) lichen planus shows strong membrane staining of both keratinocytes and dermal lymphocytes.

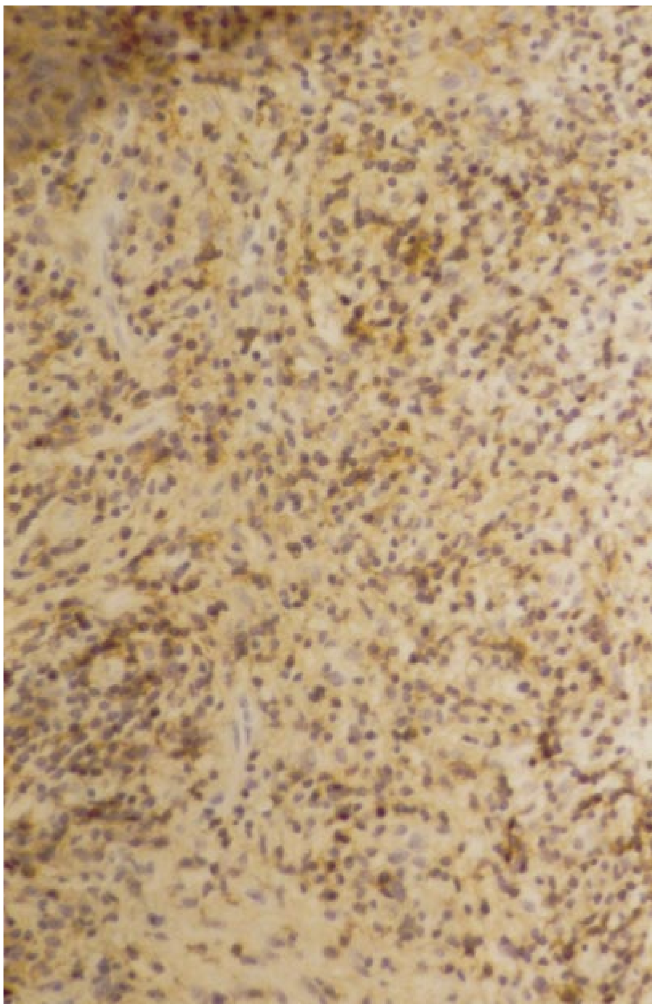

Figure 4. Analysis of Bax expression in MF by immunohistochemistry. Strong cytoplasmic staining of lymphoid cells in the dermal infiltrate.

upregulating T cell apoptosis in the skin rather than reducing the rate of T cell proliferation (Miracco *et al*, 1997), and is likely to involve Fas/Fas ligand interactions (Caricchio *et al*, 1998). (5) Mutations of Fas or Bax have been reported in other human hematologic malignancies, especially multiple myeloma (Landowski *et al*, 1997), which is characterized in the initial indolent phase by an accumulation of slowly proliferating plasma cells, and by low-grade non-Hodgkin lymphoma (Gronbaeck *et al*, 1998). (6) Demonstration of a significant decrease of Fas expression on peripheral blood $CD4^+$ lymphocytes in CTCL compared with benign lymphocytes (Dereure *et al*, 2000) and on neoplastic cells in aggressive types of CTCL (Zoi-Toli *et al*, 2000), data which overall suggest that loss of Fas receptor expression may be involved in the pathogenesis or progression of some types of CTCL. (7) The presence in peripheral blood of an abnormal population of atypical lymphoid cells that lack Fas expression in some cases of so-called primitive hypereosinophilic syndrome, a condition that can be related to a secretion of interleukin-5 by an abnormal lymphoid clone that can evolve into a peripheral T cell lymphoma (Simon *et al*, 1999). By contrast, expression of Fas by neoplastic cells did not seem to be altered in some cases of MF and SS in a recent immunohistochemistry study (Kamarashev *et al*, 1998).

Bax, the pro-apoptotic partner of bcl-2, has been found mutated in some human hematologic malignancies and colonic cancers. These mutations often involve a crucial tract of eight guanines, the deletion or insertion of one guanine leading to a frame shift, a premature stop codon, and finally to a truncated inactive molecule, no longer detectable by immunohistochemistry (Brimmell *et al*, 1998; Meijerink *et al*, 1998). In other cases, the mutations result in alterations that prevent the protein from forming efficient heterodimers with its partner bcl-2, with an imbalance between anti- and pro-apoptotic influences as a result (Meijerink *et al*, 1998). Analysis of Bax has not been reported previously in MF. Our study shows that Bax expression is conserved in the lymphoid dermal infiltrate and that no mutation is detected by PCR-SSCP. Regardless of the limitations of sensitivity of the SSCP method (Sheffield *et al*, 1993), these findings do not support a role for Bax alterations in MF. It can be pointed out that earlier studies of bcl-2 in search of gene alterations and overexpression, although rare and inconclusive, do not favor a role of bcl-2 alterations in the pathogenesis of MF either (Kanavaros *et al*, 1994; Dummer *et al*, 1995; Garatti *et al*, 1995).

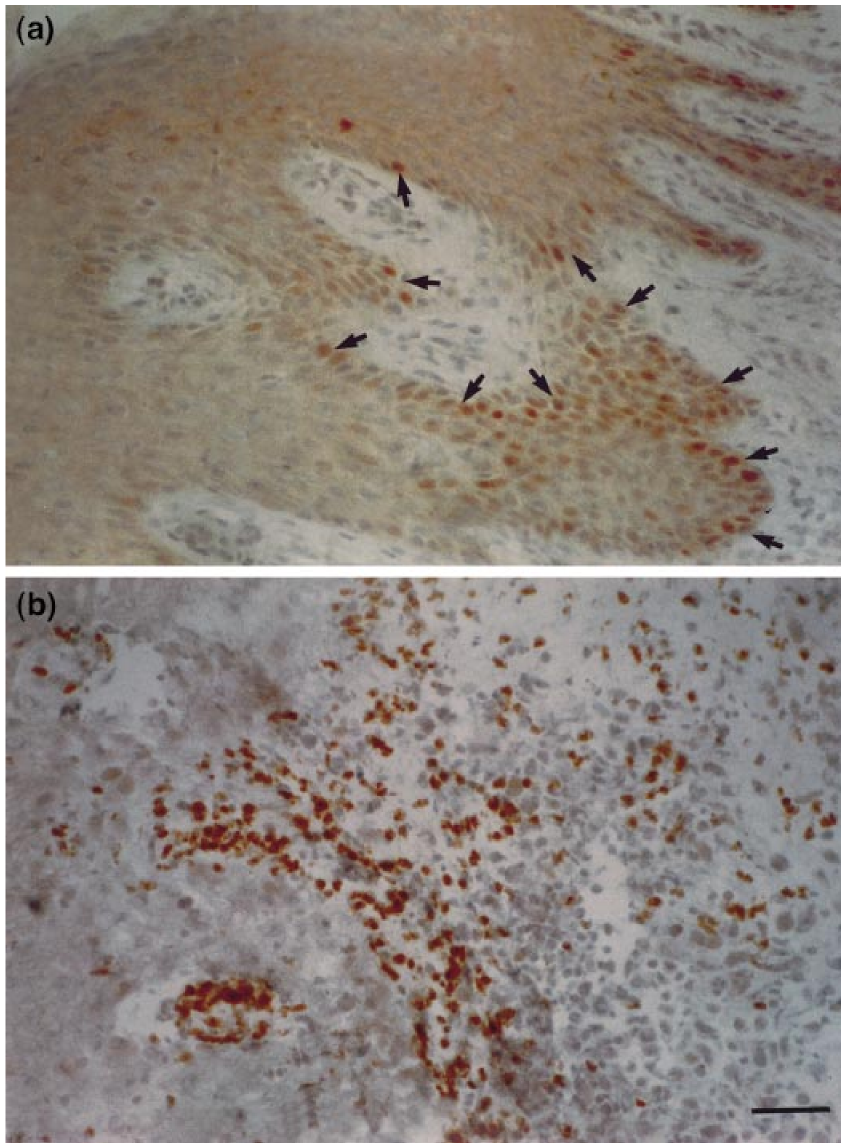

Figure 5. Analysis of p53 expression in MF. (*a*) Nuclear staining of supra-lesional, normal-appearing keratinocytes without staining of dermal lymphocytes; (*b*) nuclear staining of dermal lymphoid cells.

Molecular analysis of p53, the most frequently mutated gene in human malignancies, has been reported only four times in MF on an overall number of 81 patients from patches to tumor-stage disease, including cases transformed in large cell, more aggressive lymphoma (CTCL) (Garatti *et al*, 1995; Li *et al*, 1998; Marrogi *et al*, 1999; McGregor *et al*, 1999). Relevant mutations have been found in only 15 patients, a significant number of them (six of 15) occurring at dipyrimidine sites and resulting in C→T transitions and one case in a CC→TT tandem base transition, a mutation spectrum strikingly similar to that usually found in nonmelanoma skin cancer and characteristic of DNA damage caused by ultraviolet B radiation (McGregor *et al*, 1999). It is of interest that all reported MF cases showing p53 mutations are from advanced, tumor stages, or transformed aggressive large cell lymphoma, and that no patient with patch- or plaque-stage disease displayed p53 mutation. Our results on 41 patients with MF patches and plaques largely confirm these data. Accordingly, p53 alterations are unlikely to play a significant role in the pathogenesis of early MF. Data on p53 immunohistochemistry are more abundant. Overall, p53 staining of lymphoid cells is infrequent in MF, and is largely limited to advanced cases with either tumor stages or transformation in large cell, more aggressive lymphoma (Kanavaros *et al*, 1994; Beylot-Barry *et al*, 1995; de Misa *et al*, 1995; Garatti *et al*, 1995; Lauritzen *et al*, 1995; McGregor *et al*, 1995; van Haselen *et al*, 1997; Kim *et al*, 1998; Li *et al*, 1998; Marrogi *et al*, 1999; McGregor *et al*, 1999) with no clear relationship between molecular and immunohistochemical data (Li *et al*, 1998). Our results are consistent with these findings, showing nuclear staining in two of 11 cases that do not display any significant p53 mutation. The high expression of p53 in epidermal keratinocytes above MF dermal lesions with at least one antibody in a significant number of cases has not been previously reported and could be explained by the production of stress-inducing cytokines by neoplastic cells of the underlying dermal infiltrate. The reproducibility of this observation by others must be confirmed but this pattern was absent in all cases of eczema. If confirmed, these results could lead to a new element of discussion in the evaluation of dermal lymphoid infiltrates of uncertain nature.

In conclusion, we propose that an acquired defect in apoptosis, in some cases through alterations of Fas antigen, in a subset of activated T cells with cutaneous tropism, is involved in the pathogenesis of early MF. This defect in the ability of tumor cells to undergo apoptosis through the Fas/Fas ligand pathway could explain the common resistance of MF to chemotherapy. Conversely, two of the main regulators of apoptosis, p53 and Bax, do not seem to be involved in defective apoptosis of MF cells. Other molecules remain to be screened for functionally relevant alterations in MF, e.g., Fas ligand and Caspases. The high expression of p53 in supralesional keratinocytes remains to be

confirmed but, if so, could be an ancillary diagnostic tool in ambiguous cases of cutaneous lymphoid infiltrates.

This work was supported by grants to MEK from the American Cancer Society (grant ROG 98-125-01), the Leukemia Society of America 6178–98 and Berlex Laboratories, Richmond CA; OD was supported by grants of Fondation René Touraine, Laboratories Janssen-Cilag, the Philippe Foundation, and by the University Hospital of Montpellier France. EL was a fellow of the Lymphoma Research Foundation of America.

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