74,29 (1983)]. Monoclonal antibody to L^d (28-14-8.S) was provided by M. Lohoff (University of Erlangen, Erlangen, Germany). As an irrelevant (control) antibody for anti-L^d, a rat antibody to human glycophorin IgG was used (YTH 89.1).

26. Primary cytotoxicity and cytotoxicity after secondary in vitro restimulation were assessed as described [R. M. Zinkernagel *et al.*, *J. Exp. Med.* **162**,2125 (1985)]. Target cells were infected with either LCMV [0.1 plaque-forming units (PFU) per cell] 48 hours before the assay, or with vacc-GP (5 PFU per cell) 2 hours before the assay, or labeled with the K^b-binding LCMV-GP peptide aa33-41 (50 mM for 2 hours) [H. P. Pircher *et al.*, *Nature* **346**, 629 (1990)].

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Mutations in Fas Associated with Human Lymphoproliferative Syndrome and Autoimmunity

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Fas (also known as Apo1 and CD95) is a cell surface receptor involved in apoptotic cell death. Fas expression and function were analyzed in three children (including two siblings) with a lymphoproliferative syndrome, two of whom also had autoimmune disorders. A large deletion in the gene encoding Fas and no detectable cell surface expression characterized the most affected patient. Clinical manifestations in the two related patients were less severe: Fas-mediated apoptosis was impaired and a deletion within the intracytoplasmic domain was detected. These findings illustrate the crucial regulatory role of Fas and may provide a molecular basis for some autoimmune diseases in humans.

The life and death of lymphocytes are tightly controlled by membrane receptors that activate either proliferative or apoptotic processes (*1*). The Fas antigen has been identified as a key cell surface receptor involved in apoptotic cell death (*2*). Fas is a cell surface protein of 48 kD that plays a major role in induction of apoptosis in lymphoid cells. Mutations in the Fas-encoding gene are responsible for the lymphoproliferative disorder and associated lupuslike syndrome in *lpr* and lpr^{cg} mice (3). Defects in Fas-induced apoptosis may lead to incomplete elimination of peripheral autoreactive cells in these mice (4).

We studied three patients (two siblings and an unrelated child) with clinical and immunological features reminiscent of those seen in *lpr* mice: a lymphoproliferative syndrome, which, in one of the siblings, was associated with autoimmune hemolytic anemia, neutropenia, and thrombocytopenia (patient 3). A large proportion of peripheral blood (Table 1) and splenic T lymphocytes expressed αβ T cell receptors (TCR) but expressed neither CD4 nor CD8 molecules. These double-negative (DN) T cells, as well as the single-positive (SP) T cells showed normal expression of the TCR-CD3 complex. Analysis of TCR-Vβ utilization in DN T cells from patient 3 showed this population to be oligoclonal. The expression of Fas on activated T cells was markedly reduced in patient 1 but was comparable in patients 2 and 3 and in healthy controls (Fig. 1A). In contrast, Fas-mediated apoptosis, determined by means of hypodiploid nuclei quantification (5), was defective in T cells (Fig. 1B) and in Epstein-Barr virus–transformed B cells from all three patients: A monoclonal antibody (mAb) to Fas caused apoptosis in 65% of control cells but no detectable apoptosis in patient 1 and only 20% apoptosis in cells from patients 2 and 3. The same results were achieved with the use of the TUNEL method (6). Cells from the father but not the mother of patients 2 and 3 showed a mild reduction in Fas-mediated apoptosis (Fig. 1B), as shown with the use of both hypodiploid nucleic quantification and TUNEL methods. Staphylococcal enterotoxin B–induced apoptosis in patient 1 and antibody to CD3-induced apoptosis in patients 2 and 3 were reduced. This shows that the Fas–Fas ligand interaction does not operate in these Fas-deficient cells.

Northern (RNA) blot analysis (Fig. 2B) showed a reduction in the transcript

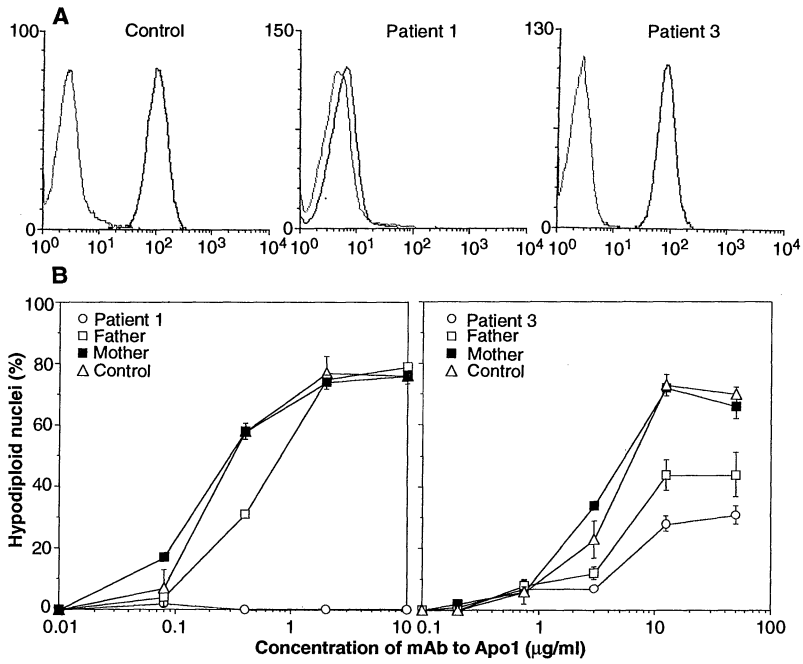

Fig. 1. (**A**) Defective Fas expression on activated T cells. (**B**) Defective Fas-mediated apoptosis. Shown is the percentage of hypodiploid nuclei (*13*) as a function of the concentration of mAb to Apo1 in cells from patients 1 and 3 (open circles), from fathers (open squares), from mothers (solid squares), and from two controls (open triangles). The same results were obtained for patients 2 and 3 in three separate experiments. The difference in sensitivity of the Fas-induced apoptosis assay between patients 1 and 3 is secondary to the use of different batches of mAb to Fas.

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size in patient 1, whereas products from patients 2 and 3 were of normal size. Fas complementary DNA (cDNA) was isolated from all patients and sequenced to determine the molecular basis of these defects. Seven primers were designed to amplify the full-length coding region (Fig. 2A). Direct sequencing of the Fas polymerase chain reaction (PCR) product (F3-R3) from patient 1 with the F4 primer revealed a 290–base pair (bp) deletion extending from nucleotides 1110 to 1400 (Fig. 3A). This deletion was responsible for the smaller transcript detected by Northern blot and resulted in the lack of the last 29 amino acids and the addition of the YKLHQE (7) peptide at position 291. This mutation extends into the death domain of the Fas molecule and is incompatible with apoptotic activity, as shown with the use of in vitro deletion mutants at position 296 (Fig. 2C) (8). Southern (DNA) blot analysis using a 3′ end-specific probe (Fig. 3B) and amplification of genomic DNA with the F4 and R3 primers resulted in single bands in control DNA. DNA from patient 3 showed a single band with a 290-bp reduction, and the DNA from both parents had the two bands, thus indicating their heterozygous status for the same mutation. This deletion results in either an unstable protein or the retention of the protein inside the cell because there is no detectable cell surface expression.

Sequencing of clones derived from PCR reactions (F2-R2) revealed a 2-bp deletion at nucleotide 1005 in clones from patients 2 and 3 and in their mother but not in clones from their father (Fig. 3C). The frame shift caused by this 2-bp deletion generates a premature stop codon at amino acid 263 in the cytoplasmic domain, which is preceded by an unrelated nine–amino acid peptide. Protein immunoblot analysis revealed two forms of the Fas protein in the patients and in their mother, one being 5 kD smaller than the other as would be predicted from the maternally derived mutation. This mutation is not compatible with apoptotic activity, as demonstrated with in vitro Fas mutants (Fig. 2C) (8). Clones corresponding to the wild-type Fas transcript inherited from the father were also found. Although we could not find any defect in the sequence of the Fas gene from the father, the clinical expression as well as the profoundly reduced Fas-mediated apoptosis found in patients 2 and 3 could not be accounted for only by the maternally inherited mutation, because this defect has no phenotypic expression in the mother. Moreover, Fas-induced apoptosis of the father's T cells was moderately reduced. This suggests that an unknown subclinical defect affecting the Fas-mediated apoptosis pathway but not directly related to the Fas gene itself was inherited from the father. In the context of a Fas mutation, this defect has clinical manifestations. Digenic autosomal recessive disorders have already been described in humans; for example, in Badet-Biedl syndrome, a recessive form of retinal dystrophy (9). Each mutation at the heterozygous level does not produce any obvious phenotype, whereas heterozygocity at both loci results in the disease.

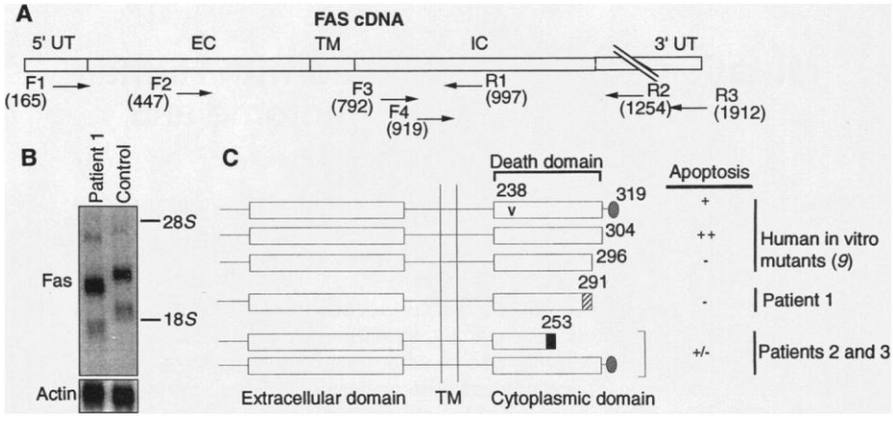

Fig. 2. Structure of Fas cDNA and Northern blot analysis. (**A**) Structure of the Fas cDNA and location of the primers used for PCR amplification (*14*). F1: 5′-TCTTTCACTTCGGAGGATTGCT-3′; F2: 5′-TGCCAAGAAGGGAAGGAGTA-3′; F3: 5′-AGAAAGCACAGAAAGGAAAACC-3′; F4: 5′-CTAAGTCAAGTTAAAGGC-3′; R1: 5′-ACTTTCTGTTCTGCTGTGTCTT-3′; R2: 5′-ACAGCCAGCTATTAAGAATC-3′; R3: 5′-TACTCTCCTGCTCAAAATGCTT-3′. UT, untranslated region; EC, extracellular; TM, transmembrane; and IC, intracytoplasmic. (**B**) Northern blot analysis of Fas on polyadenylated RNA (4 μg) from patient 1 and the control. (**C**) Summary of human mutations in the Fas gene and their effects on Fas-mediated apoptosis. In patients, 1, 2, and 3, the shaded and solid rectangles at the COOH-terminus of the protein represent the small peptide added because of the frame shift in the gene encoding Fas.

Table 1. Analysis of the patients' phenotypes. A 10-month-old girl (patient 1) born to parents who were second cousins had hydrops foetalis without evidence of infection or malformation. Hepato- and splenomegaly were noted at birth. Marked lymphocytosis up to 90 × 10^3 per microliter was detected at 5 months of age with lung infiltrates and enlarged intra-abdominal lymph nodes. Two siblings (a 12-year-old girl and a 9-year-old boy; patients 2 and 3, respectively) born to unrelated healthy parents developed splenomegaly associated with lymph node enlargement, hepatomegaly, and failure to thrive. Polyclonal hypergammaglobulinemia A and G was detected in all patients and was associated with a monoclonal immunoglobulin GI in patient 1. Autoimmune thrombocytopenia was also noticed in this patient. In patient 2, splenomegaly was diagnosed at 5 years of age, and splenectomy was carried out when she was 12 because of hypersplenism with anemia, thrombocytopenia, and mild neutropenia. Splenomegaly was detected in patient 3 at birth; hemolytic anemia and autoimmune thrombocytopenia (autoantibody to glycoprotein Ib) that occurred at 8 years of age were treated with steroids, azathioprine, and cyclophosphamide, and by splenectomy.

Cells	Patient 1	Patient 2	Patient 3	Control
	Cell counts (cells per microliter)			
Neutrophils	1,200 to 11,000	1,000 to 3,000	100 to 3,000	2,000 to 8,000
Platelets	17,000 to 190,000	60,000 to 110,000	5,000 to 100,000	150,000 to 400,000
Lymphocytes	35,000	9,300	2,800	1,500 to 4,000
	Lymphocyte phenotype (%)			
CD3	86	86	91	70 to 90
CD4	11	9	12	35 to 65
CD8	16	44	49	15 to 35
CD16	5	5	5	1 to 10
CD19	4	6	2	1 to 10
	Phenotype of CD3⁺ lymphocytes (%)			
CD2	99	100	99	98 to 100
$CD4^-CD8^-$	70	41	38	0 to 3
TCR γ/δ	4	17	21	1 to 10
TCR α/β	96	83	79	90 to 99
Human lymphocyte antigen class II to	78	46	23	0 to 10

There is an apparent gradation in the severity of the disease according to the extent to which apoptosis is affected. Patient 1, for whom no Fas expression was detectable, was the most affected with clinical symptoms that were present at birth. Fas expression was normal in patients 2 and 3, although the maternally inherited mutated allele that leads to the disruption of the Fas death domain cannot give rise to a functional protein (*8*). This mutation, together with the defect inherited from the father, leads to a profound, though less severe, defect in Fas-mediated apoptosis.

In humans, Fas is constitutively expressed in large amounts on CD4 and CD8 double-positive (DP) thymocytes and on peripheral T and B cells after activation (*10*). Consequently, lymphoproliferation as well as oligoclonality of SP and DN T cells may result from a defect in either central or peripheral Fas-dependent selection mechanisms. The accumulation of DN T cells may therefore be a consequence of an abnormal differentiation pathway or down-modulation of CD4 or CD8 molecules in the periphery as observed in *lpr* mice (*11*) . Our study defines a syndrome in humans that phenotypically and molecularly resembles the lpr syndrome in mice. Patient 1 had very limited autoimmune manifestation of the syndrome in spite of the lack of Fas expression. This seems to argue against a major role for Fas in central negative selection but for a role in controlling peripheral lymphoid responses. The genetic dependency for autoimmune manifestation has also been noted in the murine lpr mutation. Although the MRL/lpr and MRL/gld mice develop both a lymphoproliferative syndrome and autoimmune manifestations (lupuslike syndrome and nephritis), these Fas–FasL mutations in organisms with different genetic backgrounds only result in lymphadenopathy and splenomegaly (*12*). Future studies in patients with a similar syndrome will provide new insights into the role of Fas in lymphoid development and the effect of multigene allele expression in the onset of autoimmune diseases such as diabetes in *nod* mice or diabetes (and possibly systemic lupus and rhumatoid arthritis) in humans.

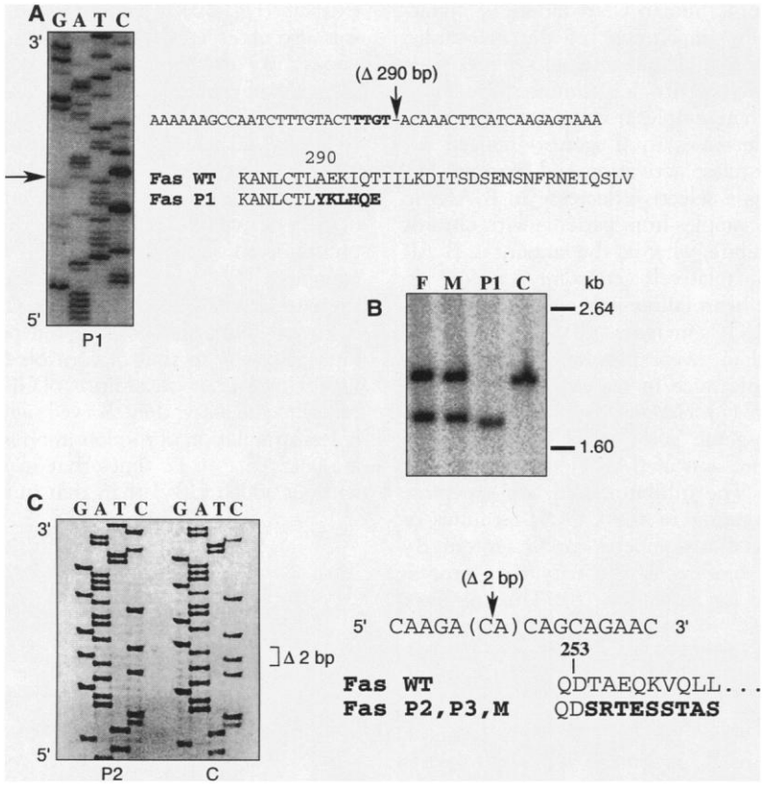

Fig. 3. The Fas molecular defect. (**A**) Direct sequencing of the PCR product (F1-R3) from patient 1 (P1) with the use of F4 primer (*14*). WT, wild type. The arrow indicates the breakpoint of the 290-bp deletion. The 290-bp deletion leads to the elimination of the last 29 amino acids and their replacement by an unrelated six–amino acid peptide (in bold). (**B**) Southern blot analysis on genomic DNA from patient 1 (P1), her parents (F and M), and a control (C) with the use of a probe specific to Fas exon 9. (**C**) Sequence analysis of PCR clones (F1-R2) from patient 2 (P2), and control (C), showing the maternally inherited 2-bp CA deletion that leads to the termination of the normal protein sequence at position 253 and the addition of an unrelated nine– amino acid peptide (in bold). WT, wild type; P2, patient 2; P3, patient 3; and M, mother.

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7. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; H, His; K, Lys; L, Leu; Q, Gln; R, Arg; S, Ser; T, Thr; and Y, Tyr.
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13. In the immunofluorescence study, T cells activated for 3 days with phytohemagglutinin (PHA) were incubated with an mAb to Fas (anti-Apo1) [B. C. Trauth *et al.*, *Science* **245**, 301 (1989)] at a final concentration of 1 mg/ml in phosphate-buffered saline supplemented with 1% bovine serum albumin for 20 min at 4°C. Cells were then washed and stained with phycoerythrein-coupled goat antibody to mouse immunoglobulin (Caltag, San Francisco, CA) and analyzed on a flow cytometer (FACSCan, Becton Dickinson, San Diego, CA). For the apoptosis assay, after activation for 6 days with PHA and interleukin 2, T cells (0.5×10^6 per milliliter) were transferred to tissue culture plates coated with mAb to Apo1 at the indicated concentration (depending on the antibody batch). Cells were washed after 24 hours and lysed in hypotonic propidium iodide (PI) (50 mg/ml in 0.1% citrate sodium and 0.1% Triton X-100) as described elsewhere (*5*) . The PI fluorescence of individual nuclei was measured with a FACStar flow cytometer (Beckton Dickinson, San Diego, CA) and the percentage of hypodiploid nuclei (less than 2N DNA content) was evaluated.
14. Total RNA from peripheral blood lymphocytes of control, parents, and patients was subjected to reverse transcriptase PCR as described [F. Rieux-Laucat, F. Le Deist, F. Selz, A. Fischer, J.-P. de Villartay, *Eur. J. Immunol.* **23**, 928 (1993)] (except for the annealing step, which was carried out at 55°C), with the use of primers described in Fig. 1A. PCR products were directly sequenced or cloned into a suitable M13 vector and several clones were sequenced. Mutations detected on clones were confirmed by direct sequencing of PCR-amplified Fas cDNA. For Southern blot analysis, genomic DNA was digested with Bam HI,and the corresponding blot was hybridized with a probe specific for Fas exon 9, obtained by PCR (F4 and R3 primers) on genomic DNA.

15. The studies were done after parental consent for the investigations was obtained and were in accordance with institutional review board approval. Supported by grants from the Association pour la Recherche sur le Cancer, INSERM, and the Deutsche Forschungsgemeinschaft (De 271/3–1). We thank F. Selz and R. de Chasseval for technical assistance and the medical and nursing staff who take care of the patients. We also thank P. Golstein for advice, D. Guy-Grand for critical reading of the manuscript, and P. Krammer for support and suggestions.

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