Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 10033–10037, December 1990
Immunology

Primary combined immunodeficiency resulting from defective transcription of multiple T-cell lymphokine genes

(lymphokine gene regulation/interleukin 2 therapy)

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Contributed by Robert A. Good, September 25, 1990

ABSTRACT **The circulating T lymphocytes of a female child with recurrent opportunistic infections were normal in number and phenotype but exhibited poor proliferation and decreased synthesis of the T-cell growth factor interleukin (IL) 2 in response to mitogens. Recombinant IL-2 fully restored the proliferative responses of her T cells, suggesting that her poor immune function was related to IL-2 deficiency. Northern blot analysis of total cellular RNA from the patient's T cells revealed markedly decreased levels of IL-2 mRNA of normal size. In addition, mRNA levels of other lymphokines selectively expressed by T cells, which include IL-3, IL-4, and IL-5, were either severely depressed or absent. The levels of interferon γ mRNA were moderately decreased, while those of granulocyte–macrophage colony stimulating factor, a lymphokine the production of which is not restricted to T cells, were unaffected. The decreased level of lymphokine mRNA in the patient's T lymphocytes was not from enhanced catabolism but resulted from a diminution in the transcription rate of the affected lymphokine genes. Normal transduction via the T-cell receptor/CD3 complex of biochemical signals necessary for the initiation of lymphokine gene transcription indicated that the defect was distal to the membrane signal-transducing apparatus. The defect is hypothesized to involve a T-cell-specific trans-acting regulatory factor required for transcription of the affected lymphokine genes.**

Cellular and humoral immune responses to antigens strictly depend on the synthesis by activated T cells of a multitude of lymphokines. These include interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, interferon γ (IFN-γ), colony-stimulating factors, tumor necrosis factor (TNF) β (lymphotoxin), transforming growth factor β, and other yet uncharacterized factors (1). Lymphokines facilitate the proliferation and differentiation of lymphocytes into effector cells, such as cytotoxic T cells or immunoglobulin-producing plasma cells. They also exert a profound influence on nonimmune cells, such as hematopoeitic cells and fibroblasts, contributing to the adaptation of the host to the ongoing immune response.

We have previously reported a child with severe combined immunodeficiency, associated with poor T-cell proliferation in response to mitogens, which was corrected both *in vitro* and *in vivo* by supplementation with recombinant IL-2 (2). We here demonstrate that, in addition to their decreased production of IL-2, the T lymphocytes of this child were also defective in the production of other lymphokines selectively expressed by T cells. These lymphokines included IL-3, IL-4, IL-5, and IFN-γ. The transcription by the patient's T cells of granulocyte–macrophage colony-stimulating factor (GM-CSF), a lymphokine the production of which is not restricted to the T-cell lineage, was normal. Also, the synthesis of the patient's peripheral blood mononuclear cells (PBMC) of TNF-α and IL-6, cytokines predominantly produced by monocytes, was unimpaired. The defective lymphokine production by the patient's T lymphocytes resulted from an impaired rate of transcription of the affected lymphokine genes. The transduction of signals via the T-cell receptor/CD3 complex, which is necessary for initiation of lymphokine gene transcription, was normal. We postulate that the defect in this patient involves a T-cell-specific trans-acting regulatory factor that helps initiate transcription of the affected lymphokine genes.

CASE REPORT

The patient is a 3-yr and 8-mo-old girl born at 37 weeks of gestation by Caesarean section to a 30-year-old mother after a pregnancy complicated by oligohydramnios. The patient did well for the first 2 mo of life. Thereafter, she developed a persistent generalized vesicular eruption after exposure to a sibling with varicella virus, which ultimately resolved after i.v. therapy with acyclovir. Between the fourth and sixth months of age and after her recovery from chicken pox, she had a normal lymphocyte count and phenotype, absent delayed-type hypersensitivity response to a battery of seven antigens, impaired *in vitro* proliferation of T cells to mitogens, hypogammaglobulinemia (IgG 170 mg/ml; IgA < 1 mg/ml; IgM 12 mg/ml), and low varicella zoster titers (IgG titer 1:16; IgM titer < 1:8). Tests for human immunodeficiency virus in the child and in the mother were negative. The activity in peripheral blood cells of adenosine deaminase and purine nucleoside phosphorylase, two enzymes of the purine salvage pathway, the deficiency of which results in a distinct form of severe combined immunodeficiency, were normal. The patient was diagnosed with primary severe combined immunodeficiency and was placed on i.v. γ globulin therapy. However, the patient suffered from progressive opportunistic infections, including oral thrush and *Pneumocystis carinii* pneumonia, and failed to thrive. At the age of 6 mo, the patient was transplanted with a T-cell-depleted haploidentical (paternal) bone-marrow graft. This graft as well as a subsequent booster graft given 81 days later were both rejected. Based on the observation that the patient's T cells failed to secrete detectable amounts of IL-2 upon stimulation with mitogens but proliferated well in response to a combination of mitogens and exogenous IL-2, the patient was started on i.v. IL-2 replacement therapy. The patient's clin-

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Abbreviations: $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; IFN-γ, interferon γ; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; mAb, monoclonal antibody.

ical status rapidly improved, and she was discharged on IL-2 therapy, which was given at 30,000 units/kg 3 times a week for 2 yr. The patient remained free of serious infections while on IL-2 therapy. Her IL-2 treatment was discontinued 1 yr ago. The patient is currently doing well clinically, supported by i.v. immunoglobulin therapy. Her lymphocytes continue to display defective proliferation and decreased IL-2 production in response to mitogens. The initial clinical course of the patient and her response to IL-2 treatment have been described elsewhere (2).

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs). The anti-CD2 mAb 9.6 and the anti-CD3 mAb Leu4 were a gift of Cox Terhorst (Dana–Farber Cancer Institute, Boston). The anti-CD28 mAb 9.3 was a gift of Carl June (Naval Medical Research Institute, Bethesda, MD). The anti-CD2 mAb OKT11 and the anti-CD3 mAb OKT3 were obtained from Ortho Diagnostic Systems. mAbs directed against CD3 (Leu4), CD4 (Leu3a), CD8 (Leu2a), and T-cell receptor α/β chains (WT31) were from Becton Dickinson.

Cells and Cell Cultures. The isolation of PBMC and their stimulation with mitogens were done as described (3). T-cell lines were generated from PBMC by periodic stimulation with the lectin phytohemagglutinin-P, irradiated allogeneic feeder cells, and activated T-cell-conditioned media, as described (4). Ninety-seven percent of the patient's cell line lymphocytes stained positive for the $\alpha\beta$ T-cell receptor, 59% were positive for CD4, and 37% were positive for CD8. The phenotype of T-cell lines derived from normal individuals closely matched that of the patient's T-cell lines.

Immunofluorescence. The expression of cell-surface markers on PBMC was monitored by flow cytometry, as described (3).

Assay of Cytokine Production. Cells were grown for 48 hr with or without the indicated stimuli. Thereafter, the culture supernatants were harvested, and their lymphokine content was assayed by using commercially available ELISA (IL-4, IL-6, and TNF-α) or RIA (IL-2 and IFN-γ) kits. IL-2 concentrations were additionally determined using a bioassay, as described (3).

RNA Preparation and Analysis. Total cellular RNA was prepared by lysing the cells with guanidine isothiocyanate followed by centrifugation of the lysate over a CsCl cushion (5). Northern blot analysis of the isolated RNA samples was done as detailed (6). Briefly, 10 μg of each RNA sample was size-fractionated by electrophoresis in 1% agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized with the appropriate [^{32}P]dCTP-labeled probe. cDNA probe for human IL-2 was obtained from Oncor (Gaithersburg, MD). Gibbon IL-3 cDNA probe was a gift of Bernard Mathey Prevot (Dana–Farber Cancer Institute, Boston). Human IL-4 cDNA probe was a gift of Ken-Ichi Arai (DNAX, Palo Alto, CA). Human IL-5 cDNA probe was a gift of Steven Clark (Genetics Institute, Cambridge, MA). IFN-γ cDNA probe was a gift of Patrick Gray (Genentech, San Francisco). Human GM-CSF oligonucleotide probe was obtained from Amgen Biologicals. β-actin cDNA probe was a gift of Cox Terhorst (Dana–Farber Cancer Institute). After hybridization, the filters were washed and exposed to Kodak XAR-5 x-ray films with DuPont intensifying screens for 1–7 days at −80°C.

Transmembrane Signaling Studies. T-cell receptor/CD3-mediated increase in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored as described (7). Protein kinase C-mediated phosphorylation of target proteins was done as detailed elsewhere (8).

Nuclear Run-on Assays. T cells either were left untreated or were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 1 hr at 37°C. Thereafter, the cell nuclei were isolated, and all newly initiated RNA transcripts contained within these nuclei were further elongated and were simultaneously radiolabeled by adding a mixture of ATP, GTP, CTP, and [^{32}P]UTP, as described (9). The radiolabeled RNA was isolated and hybridized to a nitrocellulose filter on which various lymphokine cDNA species were immobilized. After hybridization, the filters were washed and exposed to Kodak XAR-5 x-ray films with DuPont intensifying screens for 1–7 days at −80°C.

RESULTS

Flow cytometric analysis of the patient's PBMC revealed normal absolute numbers and percentages of T cells that expressed the T-cell receptor/CD3 complex (80%), CD4 (57%), and CD8 (22%). The majority of her circulating T cells (97%) expressed the α/β heterodimer form of the T-cell receptor. Table 1 shows that the patient's peripheral blood T lymphocytes failed to proliferate in response to stimulation with a mitogenic anti-CD3 mAb, OKT3, or with a mitogenic combination of anti-CD2 mAbs, OKT11 plus 9.6. The patient's T cells also proliferated poorly in response to the lectin phytohemagglutinin-P. Recombinant IL-2 corrected the defective proliferation of the patient's T cells to mitogens (Table 1), suggesting that IL-2 deficiency played a critical role in the patient's immune dysfunction. This hypothesis was confirmed by the observation that phytohemagglutinin-P-stimulated T cells from the patient failed to produce detectable levels of IL-2 as determined by a RIA (Table 2) or by a bioassay (data not shown). Moreover, treatment of the patient's PBMC with a combination of protein kinase C activator PMA and Ca^{2+} ionophore ionomycin, agents that bypass the cell-membrane signal-transduction apparatus to directly initiate cellular activation, induced only modest production of IL-2 as compared with control PBMC (Table 2). Additionally, the patient's T lymphocytes were also defective in their production of other lymphokines, the synthesis of which is restricted to T lymphocytes such as IL-4 and INF-γ. In contrast, the synthesis by the patient's PBMC of TNF-α and IL-6, cytokines that are predominantly produced by monocytes, was unimpaired (Table 2).

The defective production of lymphokines by the patient's T cells reflected a profound decrease in the level of mRNA species coding for the deficient lymphokines. Northern blot analysis of total cellular RNA derived from PBMC that have been stimulated with PMA and ionomycin demonstrated a profound decrease in the levels of IL-2 mRNA in the patient relative to control cells (Fig. 1). The mRNA levels of two other T-cell restricted lymphokines, IL-4 and IL-5, were also depressed. The levels of mRNA coding for the predominantly

Table 1. Proliferative responses of patient to mitogens

	[^{3}H]Thymidine incorporation, cpm			
	Experiment 1		Experiment 2	
Stimulus	Patient	Control	Patient	Control
Medium	538	1,911	377	1,228
Medium + IL-2	13,336	60,060	9,187	39,862
Anti-CD3 (OKT3)	720	22,680	7,134	126,550
Anti-CD3 + IL-2	67,785	198,090	224,565	276,480
Anti-CD2	673	183,487		
Anti-CD2 + IL-2	298,610	268,160		
PHA	25,583	365,690	40,080	375,465
PHA + IL-2	521,010	418,070	319,800	359,100

Recombinant IL-2 was used at 100 units/ml, all mAbs were used at 1 μg/ml, and PHA-P was used at 10 μg/ml. Results shown are the means of triplicate determinations. Similar results were found in five other experiments.

Table 2. Patient lymphokine and monokine synthesis

	Patient			Control		
	Medium	PHA	PMA/ionomycin	Medium	PHA	PMA/ionomycin
IL-2, fmol/ml	ND	ND	69	ND	90	2745
IFN-γ, units/ml	<1.5	22	75	<1.5	310	782
IL-4, pg/ml	ND		3	ND		110
IL-6, ng/ml	2.2		29	2		30
TNF-α, pg/ml	1.1		400	1.8		370

PMA was used at 20 ng/ml and ionomycin at 0.5 μM. Similar results were found in three other experiments. ND, not detectable.

monocytic cytokine IL-6 were unaffected (Fig. 1). These results indicated that the patient's T lymphocytes were selectively affected by a defect that impaired their capacity to synthesize multiple lymphokines.

Because of the limitations on the number of fresh lymphocytes that could be obtained at any one time from the patient and to facilitate in-depth analysis of the defect in the patient's T lymphocytes, we established continuously growing T-cell lines from the patient's peripheral blood lymphocytes. These cell lines were similar to the patient's peripheral blood T cells, both in their phenotype and in their defective production of lymphokines after their stimulation with mitogens. Northern blot analysis of total cellular RNA isolated from these cell lines after activation with PMA and ionomycin confirmed the existence of multiple lymphokine deficiency state (Fig. 2). Specifically, the levels of IL-2 mRNA were greatly decreased in patient versus control T-cell lines. mRNA coding for IL-4 and IL-5 was barely detectable, while the levels of mRNA coding for IL-3 were markedly depressed. IFN-γ was also affected but to a lesser extent. In contrast, the levels of mRNA coding for GM-CSF, a cytokine not restricted in its expression to T cells, were normal. The size and the time course of accumulation of each of the lymphokine gene transcripts affected by the disease process was normal. The multiple lymphokine deficiency seen in the patient's T cells was not a consequence of a primary IL-2 deficiency. This fact was suggested by the failure of recombinant IL-2 supplementation to upregulate the expression of IL-3 and IL-4 upon stimulation of the patient's T cells with PMA and ionomycin (data not shown).

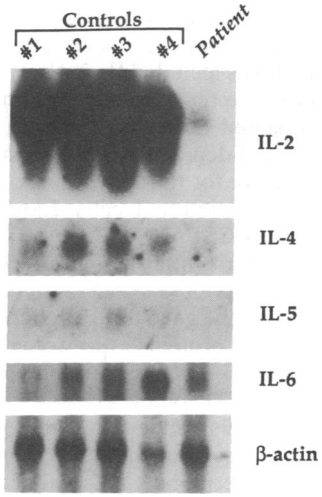

FIG. 1. Expression of lymphokine mRNA in patient and in control PBMC stimulated with PMA at 20 ng/ml and 0.5 μM ionomycin for 3 hr. Total cellular RNA was fractionated on 1% agarose-formaldehyde gel, transferred to a nitrocellulose filter, and probed for the respective lymphokine as described. The same filter was subsequently probed with [^{32}P]dCTP-labeled β-actin cDNA, which was used as control probe to determine the relative amount of RNA in each lane.

The defective synthesis of lymphokine mRNA was not due to an abnormality in the early activation events that follow the engagement of the T-cell receptor/CD3 by mitogens and that are necessary for the initiation of lymphokine gene transcription. Both Ca^{2+} mobilization and protein kinase C activation, two key activation events required for induction of lymphokine gene transcription in T lymphocytes (10), were similarly induced in patient and control T lymphocytes treated with mitogens (Fig. 3). Also, second messengers were similarly generated in patient and control lymphocytes in the wake of T-cell receptor/CD3 engagement (data not shown). These results indicated that the defect was localized at a site distal to the signal-transducing apparatus.

The decrease in lymphokine mRNA levels could have resulted from a decreased rate of initiation of transcription of these genes. Alternatively, it could have resulted from enhanced degradation of the mRNA species, which may have been otherwise transcribed at a normal rate. Agents that interfere with lymphokine mRNA degradation, such as cycloheximide, an inhibitor of protein synthesis (11), and mAb

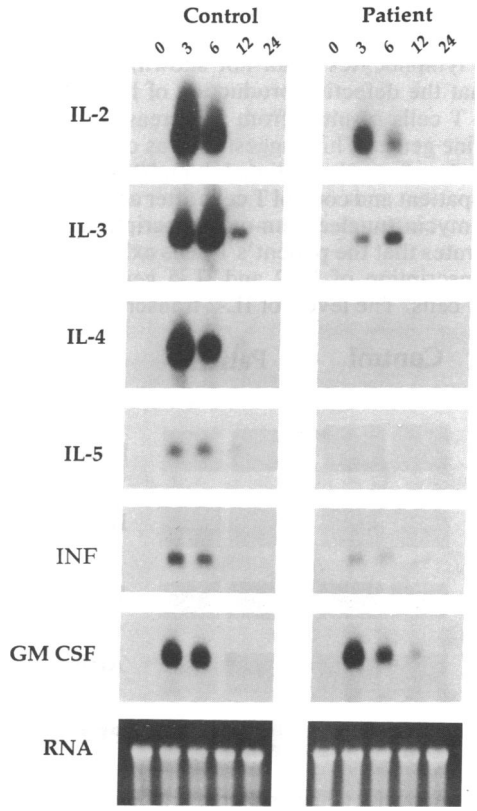

FIG. 2. Expression of lymphokine mRNA in patient and in control T-cell lines stimulated with PMA at 20 ng/ml and 0.5 μM ionomycin for the indicated periods (in hr). Ten micrograms of total cellular RNA per sample was fractionated on 1% agarose/formaldehyde gel, transferred to nitrocellulose filters, and probed for the respective lymphokine, as described.

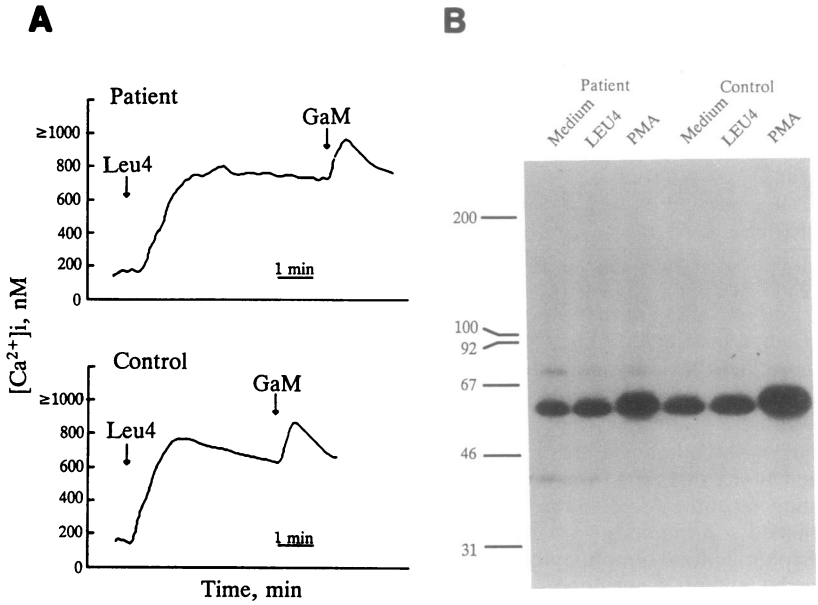

FIG. 3. (*A*) Elevation of $[Ca^{2+}]_i$ triggered via the T-cell receptor/CD3 complex in patient and in control T-cell lines. Lymphocytes were loaded with the acetoxymethyl ester of the Ca^{2+}-sensitive dye quin-2 at 5 μM for 15 min at 37°C. Thereafter the cells were washed, treated with the anti-CD3 mAb Leu4 at 1:100 dilution of ascitic fluid, and monitored for Ca^{2+}-induced fluorescence signals by using a LS-5 fluorimeter (Perkin–Elmer). The Ca^{2+}-mobilization response to crosslinking of T-cell receptor/CD3 complex was determined by further adding goat anti-mouse IgG antibodies (GαM). Calculation of $[Ca^{2+}]_i$ was done as described (7). (*B*) Phosphorylation of the T-cell surface antigen CD5 induced by anti-CD3 mAb Leu4 or by PMA in patient and in control T-cell lines. Lymphocytes were loaded with [^{32}P]orthophosphate at 1 mCi/ml for 3 hr at 37°C. The cells were then washed free of [^{32}P]orthophosphate and treated with mAb Leu4 at 1:100 dilution of ascitic fluid or with PMA at 50 ng/ml for 15 min at 37°C. Cells were subsequently lysed, and CD5 was immunoprecipitated, electrophoresed, and visualized as described (8). Relative phosphorylation intensity of CD5 in each lane was determined by densitometry and was as follows: for patient medium 100, Leu4 140, and PMA 300; for control medium 100, Leu4 145, and PMA 340.

9.3, directed against the T-cell surface protein CD28 (12), failed to correct the lymphokine deficiency observed in the patient's lymphocytes (data not shown). This finding suggested that the defective production of lymphokines by the patient's T cells resulted from a decreased transcription of lymphokine genes. This suggestion was confirmed by examining levels of newly initiated lymphokine transcripts in nuclei of patient and control T cells after activation with PMA and ionomycin (nuclear run-on transcription assay). Fig. 4 demonstrates that the patient's T cells exhibited defective *de novo* transcription of IL-2 and IL-5 genes compared with normal T cells. The levels of IL-2 transcripts were markedly

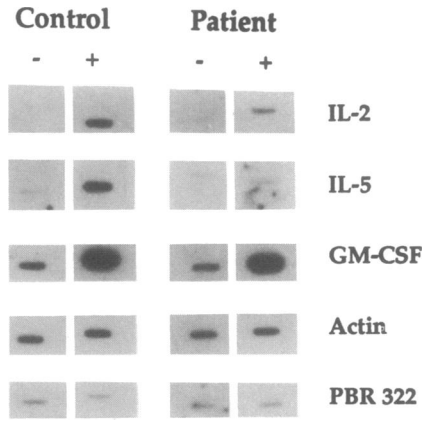

FIG. 4. Assay for initiation of transcription of lymphokine genes in patient and control T-cell lines either left untreated (−) or stimulated with PMA and ionomycin for 1 hr (+). Nuclear RNA was prepared, labeled, and after equalization for number of counts in patient and control samples, was probed for respective lymphokine transcripts as described. Transcripts of noninducible β-actin gene were used as positive control, and plasmid pBR322 DNA was used to measure nonspecific hybridization.

depressed, whereas IL-5 transcripts were undetectable. In contrast, the level of GM-CSF transcripts were unaffected. These results indicated that the defect in the patient's lymphocytes is related to an abnormality affecting the initiation of transcription of lymphokine genes upon T-cell activation rather than enhanced lymphokine mRNA degradation.

DISCUSSION

T lymphocytes of a child with primary severe combined immunodeficiency were severely defective in the production of several lymphokines that are selectively expressed in T cells. These lymphokines included IL-2, IL-3, IL-4, IL-5, and IFN-γ. The defective expression of these lymphokines after T-cell activation resulted from decreased transcription rate and not from enhanced degradation of lymphokine mRNA. The defect was selective for these cytokines, the production of which is largely restricted to T cells. Expression of other cytokines, such as GM-CSF, TNF-α, and IL-6, all of which are not restricted in their synthesis to the T-cell lineage, was unaffected.

Induction of lymphokine gene transcription is mediated by a set of DNA-binding proteins, the activity of which is regulated by biochemical signals generated upon engagement of the T-cell receptor/CD3 complex by antigens or mitogens. These proteins bind to distinct regulatory sequences located upstream from the transcription initiation site, resulting in the initiation of gene transcription (13). A defect in the T-cell receptor-coupled signal-transduction apparatus can cause defective expression of lymphokine genes, similar to what we have described (3) for another patient with primary immunodeficiency. However, several lines of evidence suggest that this is not the case for this patient. Both Ca^{2+} mobilization and protein kinase C activation, the two key biochemical signals required for lymphokine gene transcription, were normally triggered in the wake of T-cell receptor/CD3 en-

gagement by mitogens. Also, bypassing the signal-transducing apparatus with PMA and ionomycin failed to correct the defect in lymphokine gene expression. These results indicated that the defect in the patient's T lymphocytes lies distal to the signal-transducing apparatus on the T-cell surface.

The defective transcription of lymphokine genes in the patient's T cells is remarkably similar to what is observed in normal T cells treated with the immunosuppressive agent Cyclosporin A. Cyclosporin A selectively inhibits the transcription of multiple T-cell lymphokines, including IL-2, IL-4, and IFN-γ (14). Cyclosporin A does not interfere with the signal-transduction apparatus of T lymphocytes. Rather, it inhibits activity of some of the DNA-binding proteins mediating transcriptional activation of lymphokine genes (15). A particular target of Cyclosporin A action is the nuclear factor NFAT-1, a T-cell-specific DNA-binding protein that is critical in initiating transcription of lymphokine genes (13). It is likely that the defect in the patient's lymphocytes may similarly involve a T-cell-specific regulatory factor such as NFAT-1.

The causal relationship between defective lymphokine production by the patient's lymphocytes and her immunodeficiency state was highlighted by the ability of recombinant IL-2 to correct the poor *in vitro* proliferative responses of her lymphocytes to mitogens and by the improvement in the clinical status of the patient after IL-2 replacement therapy. Interestingly, despite the defective expression of multiple lymphokines, replacement therapy with IL-2 alone was sufficient to restore much of her immune function (2). This effect is probably a reflection of the pivotal role this lymphokine plays in the maintenance of normal immune function. The function of other lymphokines, such as IL-3, is to some extent redundant with other cytokines, such as colony-stimulating factors (e.g., GM-CSF), the production of which is apparently little affected in this patient. This fact would account for the normal hematopoeisis seen in this patient.

Finally, the defective production of lymphokines by T lymphocytes of this patient and the resultant immunodeficiency state point to abnormalities affecting lymphokines or lymphokine receptors as another cause of immunodeficiency. This interpretation is supported by the recent identification of two patients with severe combined immunodeficiency disease with an underlying IL-2 deficiency (16, 17). Elucidation of the mechanisms underlying this subset of immunodeficiency diseases would help provide a rational, individualized approach to therapy.

We are indebted to Dr. Richard Miller, Department of Pathology, Boston University School of Medicine, Boston, MA, for IL-2 bioassay and to Dr. Fred Rosen for critical review of this manuscript. This work was supported, in part, by grants from the National Institutes of Health (1P01AI28046, AI22360 and AG05628) and from the Hood Foundation.

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