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Defective expression of early activation genes in cartilage-hair hypoplasia (CHH) with severe combined immunodeficiency (SCID)

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SUMMARY

Cartilage-hair hypoplasia (CHH) is an autosomal recessive disease of unknown etiology characterized by metaphyseal dysostosis, unpigmented hair, and defective cellular immunity. We studied peripheral blood mononuclear cells (PBMC) of a boy with CHH and combined immunodeficiency in an attempt to characterize further the immune defect in this disease. Stimulation of his PBMC with mitogens was associated with severely depressed IL-2 and interferon-gamma (IFN-γ) synthesis and IL-2 receptor α-chain (IL-2Rα) expression and resulted in poor lymphocyte proliferation that was only modestly upregulated by the addition of recombinant IL-2 (rIL-2). The defective proliferation and lymphokine synthesis were not corrected by the addition of phorbol myristate acetate (PMA) and ionomycin, agents that bypass receptor-mediated signalling, indicative of a distal abnormality. Importantly, the levels of mRNA encoding c-myc, IL-2Rα, IL-2 and IFN-γ were markedly decreased in patient lymphocytes stimulated with PMA + ionomycin as compared to control lymphocytes The defect in the expression of these early activation genes was selective in that induction by mitogens of mRNA encoding other early activation gene products such as c-fos and c-jun was not impaired. These results suggest that the underlying defect in this patient and perhaps others with CHH may be an abnormality in a component of intracellular signalling pathways or in a *trans*-acting factor which regulates the expression of a selected number of early activation genes.

Keywords immunodeficiency dwarfism cartilage-hair hypoplasia

INTRODUCTION

Cartilage-hair hypoplasia (CHH) is a rare autosomal recessive disorder of short-limbed dwarfism that occurs most frequently in Amish and Finnish populations [1,2]. Affected individuals present characteristic metaphyseal dysostosis as well as fine, fragile, sparse and unpigmented hair. Impaired immunity is an integral part of the CHH syndrome. The immunological defects range from mild lymphopenia and reduced lymphoproliferative response, to a severe combined immunodeficiency (SCID) syndrome [2,3]. A proliferative defect in B cells, T cells and fibroblasts has been also demonstrated in healthy subjects with CHH. It has been hypothesized that the defect in this syndrome could be an abnormality in cellular activation and proliferation [4]. While the identity of the gene affected in this syndrome is unknown, a recent study on Finnish patients with CHH mapped its location to chromosome 9 [5].

We studied a 9-year-old boy affected by CHH. Stimulation of the patient's peripheral blood mononuclear cells (PBMC) with antigens, mitogens or anti-CD3 MoAb failed to induce lymphocyte proliferation, IL-2 and IFN-γ synthesis and IL-2R expression. The lymphoproliferative defect and the impaired IL-2 and IFN-γ synthesis were not corrected *in vitro* by the addition of PMA and ionomycin, agents that bypass receptor-mediated signalling. Lymphocyte proliferation was only modestly restored by addition of rIL-2. In this study we investigated the molecular basis of the T cell impairment by analysing the expression of mRNA of genes involved in the early steps of T cell activation. We here report that lymphocytes of this child exhibited a selective defect in the expression of mRNA of some activation genes, including those encoding c-myc, IL-2Rα chain, IL-2 and IFN-γ. Based on these results, we hypothesize that the defect in CHH involves a step that regulates the transcription of a selected number of early activation genes such as a component of intracellular signalling pathways or a transcriptional factor.

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MATERIALS AND METHODS

Patient

The patient was a 9-year-old boy who was born, following a full-term pregnancy and normal vaginal delivery, with stigmata of CHH that included short-limbed dwarfism and fine, sparse and unpigmented hair. The child's family history was negative for CHH or for other forms of primary immunodeficiency diseases. At 6 months of age he started suffering from recurrent upper respiratory tract infections, ear infections, and persistent diarrhoea with malabsorption. He went on to develop multiple episodes of bacterial sepsis with organisms that included *Klebsiella pneumoniae*, *Streptococcus fecalis*, and *Staphylococcus epidermidis*. He also developed other infectious problems including recurrent sinusitis that required surgical drainage, and chronic oral thrush. His stool cultures were frequently positive for *Clostridium difficile*, rotavirus and adenovirus. He was started on intravenous gammaglobulin replacement therapy which controlled his upper respiratory tract and ear infections. His diarrhoea and malabsorption problem persisted however, necessitating initiation of intravenous hyperalimentation therapy. He also went on to develop recurrent vesicular eruptions due to varicella which required continuous therapy with acyclovir for its suppression. In view of his progressively worsening medical problems secondary to immunodeficiency, he underwent a haploidentical bone marrow transplant with donor marrow from his sister. He died of complications of severe graft-*versus*-host disease which developed following the transplant.

Repeated investigation of his immune function revealed the presence of absolute lymphopenia with the lymphocyte count typically in the range of 200–300 lymphocytes/mm^3. He had absent delayed type hypersensitivity response to a battery of antigens, and impaired *in vitro* proliferation of T cells to mitogens. Investigation of his humoral immunity revealed normal IgG and IgM levels and a moderately elevated IgA level (635 mg%, 108 mg%, and 246 mg%, respectively). His antigen specific antibody responses were either low or absent. There was normal 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 SCID.

Methods

Immunofluorescence. The expression of cell surface markers was monitored by flow cytometry as described in [6].

Cell isolation and proliferation. The isolation, stimulation with mitogens and ^{3}H-thymidine incorporation of PBMC, were carried out as described in [6]. PHA lines were prepared and maintained as described in [6].

Assay for lymphokine production. PBMC were grown for 48 h with or without the indicated stimuli, then the culture supernatants were harvested and IL-2 and IFN-γ content was determined by using commercially available RIA kits (IL-2: Amersham (Arlington Heights, IL); IFN-γ: Centocor (Malvern, PA).

RNA preparation and analysis. Total cellular RNA was prepared by lysis of lymphocytes with guanidinium isothiocyanate followed by centrifugation over a CsCl cushion [7]. Northern blot analysis was carried out as follows: 5 or 10 μg of the isolated RNA samples were size-fractionated by electrophoresis in 1% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized with the appropriate ^{32}P-dCTP-labelled probe. The filters were then washed and subjected to autoradiography.

Probes. IL-2 and *c-myc* cDNA probes were purchased from Oncor (Gaithersburg, MD). IFN-γ cDNA was a kind gift from P. Gray (Genentech, San Francisco, CA), IL-2Rα cDNA was a kind gift from W. C. Greene and W. J. Leonard (NIH, Bethesda, MD), *c-fos* and *c-jun* cDNA probes were a generous gift of L. H. Glimcher (Harvard School of Public Health, Boston, MA).

RESULTS

Flow cytometric analysis of the patient's PBMC revealed an abnormal number and percentage of T cells (Tables 1 and 2). More than 50% of his T cells were CD4$^-$CD8$^-$ and expressed the γ/δ heterodimer form of the T cell receptor (TCR). The patient was severely deficient in CD3$^+$CD8$^+$ T lymphocytes; almost all the T cells expressing the α/β heterodimer form of the TCR were CD4$^+$ while more than 90% of his circulating CD8$^+$ cells were CD3$^-$CD16$^+$ natural killer (NK) cells (Table 1). The latter cell population was markedly expanded, accounting for 30% to 50% of all his circulating lymphocytes. The percentage of his circulating B lymphocytes was normal.

The patient's lymphocytes proliferated poorly in response to mitogens that engage cell surface receptor molecules such as the lectin phytohaemagglutinin (PHA), the anti-CD3 MoAb OKT3, and the staphylococcal superantigen TSST-1. The patient's lymphocytes also proliferated poorly when treated with PMA and the calcium ionophore ionomycin, agents which bypass the signal transducing apparatus on the cell surface to directly activate intracellular signalling pathways. This indicated that the defect in the patient's lymphocytes lies distal to lymphocyte membrane receptors (Table 3).

We next investigated whether the defective proliferative

Table 1. Phenotypic characterization of circulating patient lymphocytes (%)

Surface marker	Patient	Control
TCR$\alpha\beta$	24	55–70
TCR$\gamma\delta$	28	2–10
CD3	51	66–75
CD3$^+$/CD4$^+$	23	35–50
CD3$^+$/CD8$^+$	2	18–30
CD3$^-$/CD8$^+$	21	3–5
CD3$^+$/IL-2Rα^+	3	<2
CD3$^+$/HLA DR$^+$	5	<2
CD19$^+$/HLA DR$^+$	6	5–10
CD16 (NK cells)	34	5–15

Surface marker expression by freshly obtained patient PBMC were determined by flow cytometry as described. The values provided are from one representative determination. Mean control values for aged matched individuals at our institution are shown on the right. Patient TCR$\gamma\delta^+$ T lymphocytes were separately determined to be exclusively CD4$^-$ CD8$^-$. Almost all of patient's CD8$^+$ lymphocytes are CD3$^-$ CD16$^+$ NK cells.

Table 2. Expression of activation antigens by patient and control PBMC following mitogenic stimulation (% positive cells)

	Patient	Control
$CD3^+/IL\text{-}2R\alpha^+$ (48 h medium)	3	6
$CD3^-/IL\text{-}2R\alpha^+$ (48 h medium)	1	2
$CD3^+/IL\text{-}2R\alpha^+$ (48 h PHA)	2·5	82
$CD3^-/IL\text{-}2R\alpha^+$ (48 h PHA)	2·5	9·5
$CD3^+/IL\text{-}2R\alpha^+$ (48 h anti-CD3)	3	50
$CD3^-/IL\text{-}2R\alpha^+$ (48 h anti-CD3)	5	3

Cells were stimulated with the mitogen shown for 48 h, then examined by flow cytometry for the expression of the IL-2Rα, a known T cell activation antigen. Similar results were found in one other experiment.

responses to mitogens was due to an abnormality in the patient's lymphocytes affecting the production of or the response to lymphokines. The patient's T lymphocytes were found to be severely defective in their production of the T cell lymphokines IL-2 and IFN-γ upon stimulation with mitogens (Table 4). However, unlike patients with lymphokine deficiency syndromes whose proliferative responses to mitogens are normalized by the addition of rIL-2 [6,8,9], those of this patient's lymphocytes were only modestly upregulated by the addition of rIL-2 and then only for some mitogens (e.g. OKT3 and TSST-1) but not others (e.g. PHA and PMA + ionomycin) (Table 3). This indicated that the depressed proliferative responses of the patient's lymphocytes could not be accounted for by the failure to produce lymphokines. In support of this conclusion was the observation that the patient's lymphocytes were also markedly

Table 3. Proliferative responses of patient and control peripheral blood mononuclear cells to mitogens

	ct/min	
Stimulus	Patient	Control
Medium	180	260
Medium + IL-2	6500	24 830
PHA-P	37 000	327 900
PHA-P + IL-2	33 000	485 000
Anti-CD3 (OKT3)	50	30 724
Anti-CD3 (OKT3) + IL-2	12 000	85 888
PMA + ionomycin	7270	135 210
PMA + ionomycin + IL-2	17 200	139 080
TSST-1	5100	82 000
TSST-1 + IL-2	18 200	78 000

Cells were treated with either the lectin PHA (10 μg/ml), the mitogenic anti-CD3 MoAb OKT3 (1 μg/ml), the staphylococcal superantigen TSST-1 (1 μg/ml), or with a combination of PMA (20 ng/ml) and ionomycin (0·5 μM). Cultures were grown in the absence or presence of rIL-2 (100 U/ml), as indicated. Following culture for 72 h, the cells were pulsed overnight with 1 μCi ^{3}H-thymidine. The cells were then harvested and their incorporation of ^{3}H-thymidine determined by scintillation counting. Results are expressed as means of triplicate determinations. s.e.m. were less than 15% of the respective mean values.

Table 4. Lymphokine production by mitogen-stimulated patient and control peripheral blood mononuclear cells

	IL-2 (fmoles/ml)		IFN-γ (U/ml)	
Stimulus	Patient	Control	Patient	Control
Medium	ND*	ND	ND	0·57
PHA	ND	40	0·56	583
PMA + ionomycin	ND	2400	12	1202
Anti-CD3	ND	ND	0·11	26
TSST-1	ND	68	0·24	450

PBMC were cultured for 48 h at 10^6 cells/ml in the absence or presence of PHA (10 μg/ml), PMA (20 ng/ml) and ionomycin (0·5 μM), the anti-CD3 MoAb OKT3 (1 μg/ml), or with the staphylococcal superantigen TSST-1 (1 μg/ml). Culture supernatants were then harvested and assayed for their IL-2 and IFN-γ content. ND, not detected.

defective in their upregulation of IL-2 receptor (IL-2R) α-chain expression following activation with mitogens (Table 4). This indicated that the defect in the patient's lymphocytes was not restricted to lymphokine production but extended to include the expression of other lymphocyte activation products such as the IL-2Rα chain.

The defect in the patient's lymphocytes not only impaired the expression of lymphokines and lymphokine receptor components but extended to include other early activation genes such as *c-myc*. Figure 1 demonstrates that while stimulation with PMA and ionomycin induced the accumulation of IL-2Rα chain and *c-myc* mRNA in control PBMC, the levels of these two transcripts remained virtually undetected in patient PBMC. Figure 2 demonstrates that the patient PBMC were also defective in their expression of IL-2 and IFN-γ mRNA, consistent with the depressed production by patient PBMC of the respective protein product (Table 4). However, the defect in the patient's lymphocytes did not impair the transcription of other early activation genes such as those encoding *c-fos* and *c-jun*. Mitogenic stimulation of patient lymphocytes resulted in effective induction of mRNA of both *fos* and *jun* . The mRNA levels of both oncogenes peaked at similar time points after stimulation in both patient and control PBMC, and the time course of oncogene induction agreed with previously published reports [10]. However, mRNA levels of both oncogenes were higher and more sustained in patient as compared to control PBMC (Fig. 1).

Because of the limitations on the number of fresh lymphocytes that could be obtained from the patient, and to facilitate the analysis of the defect in the patient's T lymphocytes, we established primary T cell lines from the patient's PBMC which were driven by periodic stimulation with the mitogen PHA. The patient T cell lines were difficult to establish and grew very slowly as compared to control T lymphocytes. Northern blot analysis of total cellular RNA isolated from these cell lines following activation with PMA and ionomycin confirmed the existence of a defect affecting the expression of multiple early activation genes. Figure 2 demonstrates that the expression of transcripts encoding c-myc, IL-2 and IFN-γ was severely depressed in the patient's T cell lines compared to control and in a manner comparable to what is seen with patient

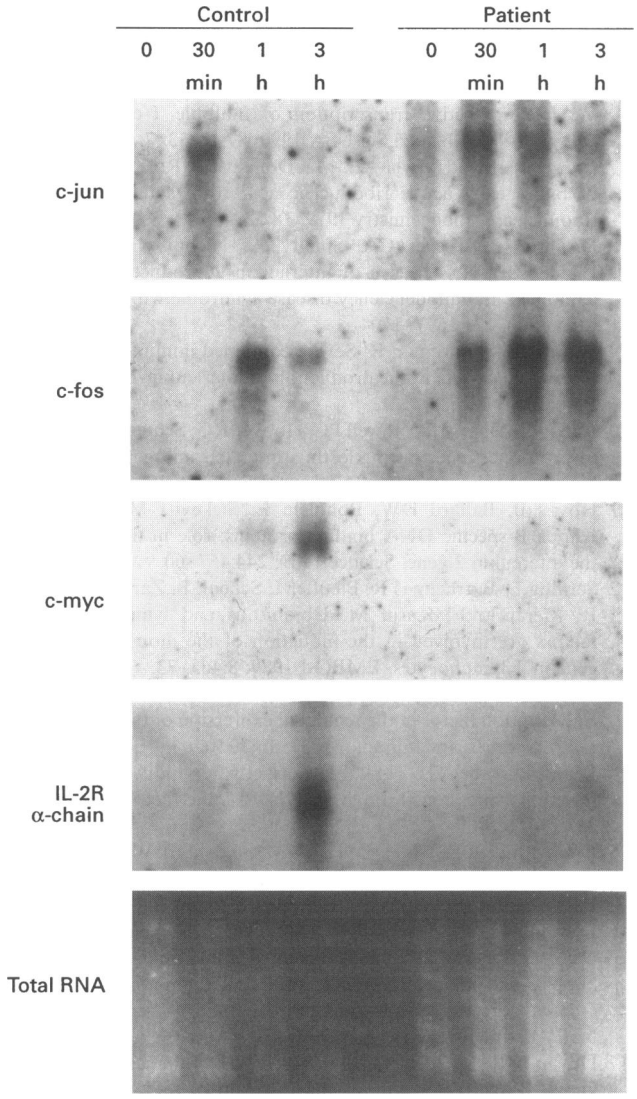

Fig. 1. Accumulation of *c-fos*, *c-jun*, *c-myc* and IL-2 mRNA in mitogen-activated patient and control PBMC. Total RNA was extracted from patient and control PBMC stimulated with PMA (20 ng/ml) and ionomycin (0·5 μM) for 30 min, 1 h and 3 h. RNA samples (5 μg/lane) were size-fractionated by electrophoresis in 1% agarose-formaldehyde gel, and transferred to a nitrocellulose filter. The same filter was sequentially hybridized with *c-fos*, *c-jun*, *c-myc* and IL-2R cDNA probes as described in Materials and Methods. Equal amounts of RNA were present in each of the respective lanes as judged from the appearance of the ethidium bromide-stained 18S and 28S RNA bands in these lanes (lower panel). The molecular weight of each of the represented bands has been verified to correspond to the known molecular weight of the respective mRNA species. The minor variation in the apparant molecular weight of some of the bands is due to small differences in the migration of the respective RNA samples in the gel.

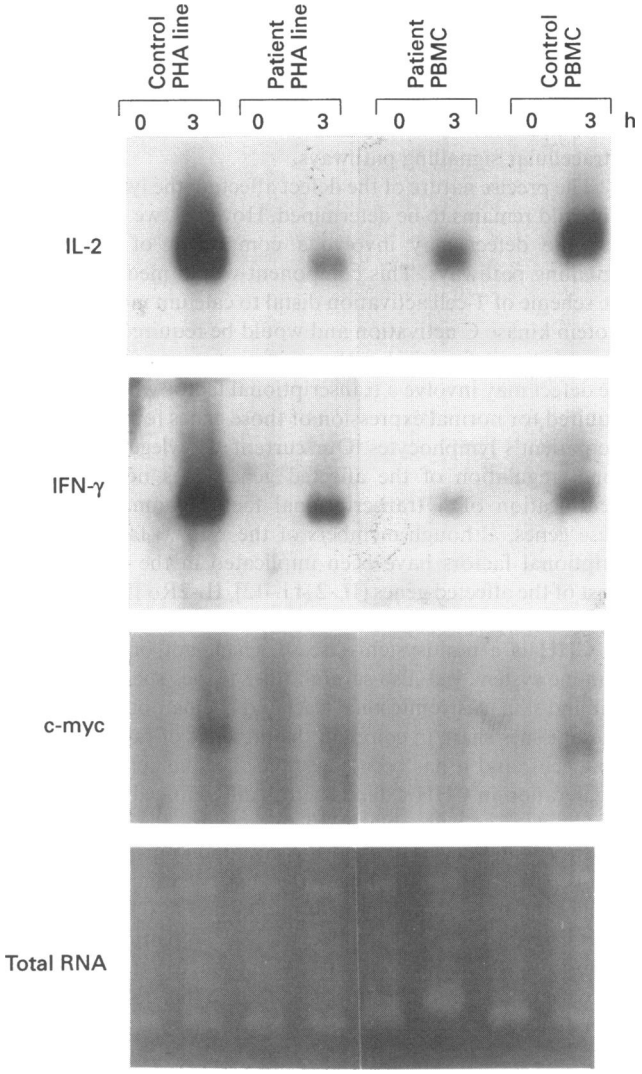

Fig. 2. Expression of IL-2, IFN-γ and *c-myc* mRNAs in patient and control T cells. PBMC and T cell lymphoblasts from PHA-driven cell lines were stimulated with PMA (20 ng/ml) and ionomycin (0·5 μM) for 3 h. Total RNA of each cell population was then extracted, size-fractionated by electrophoresis in 1% agarose-formaldehyde gel and transferred to a nitrocellulose filter. The same filter was sequentially hybridized with cDNA probes for IL-2, IFN-γ and *c-myc* as detailed in Materials and Methods. Relatively equal amounts of RNA were present in each of the respective lanes as judged from the appearance of the ethidium bromide-stained 18S and 28S RNA bands in these lanes (lower panel). The molecular weight of each of the bands shown has been verified to correspond to the known molecular weight of the respective mRNA species. The minor variation in the apparent molecular weight of some of the bands is due to small differences in the migration of the respective RNA samples in the gel.

versus control PBMC. Overall, these results indicated that the patient's T lymphocytes were affected by a defect that impaired their capacity to transcribe selected early activation genes.

DISCUSSION

T lymphocytes of our patient with CHH and associated SCID were severely defective in their expression of mRNA of several early activation genes, including those encoding IL-2, IFN-γ IL-2Rα chain, and *c-myc*. Induction of mRNA of other early activation genes such as those encoding *c-fos* and *c-jun* were not impaired, indicating that the defect in the patient's lymphocytes specifically impaired the induction of some early activation genes but not others. The poor expression by patient T lymphocytes of some of the early activation genes was associated with decreased proliferation and virtual lack of lymphokine

production in response to mitogenic stimulation. Treatment with mitogens that directly activate intracellular signalling pathways did not correct the observed abnormalities, indicating that the defect was unlikely to involve cell surface receptor molecules or associated proteins that couple these receptors to intracellular signalling pathways.

The precise nature of the defect affecting the lymphocytes of this child remains to be determined. However, we can speculate that the defect may involve a component of intracellular signalling pathways. This component would mediate a step in the scheme of T cell activation distal to calcium mobilization or protein kinase C activation and would be required for effective transcription of several early activation genes. Alternatively, the defect may involve a transcriptional factor whose activity is required for normal expression of those genes found affected in the patient's lymphocytes. Our current knowlege of transcriptional regulation of the affected genes does not permit the identification of a transcriptional factor common to all of these genes, although members of the NF-κB family of transcriptional factors have been implicated in the regulation of most of the affected genes (IL-2 [11–13], IL-2Rα [14–16], and *c-myc* [17,18]).

CHH is a multisystem disease which not only affects the immune system, but also several other tissues such as cartilage, hair and skin, gastrointestinal tract and haematopoetic elements. These tissues share in common the presence of rapidly proliferating cells, and it has been suggested that the defect in cellular proliferation in CHH extends across different cell types such as lymphocytes and fibroblasts. In this regard, the failure of the patient's lymphocytes to express *c-myc* upon mitogenic stimulation is particularly intriguing. *c-myc* has been implicated in the proliferation of several cell lineages affected in CHH, including chondrocytes [19] and lymphocytes [20]. Impairment of *c-myc* expression would provide an explanation for the generalized defect in cellular proliferation described in this disease.

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