**T Cell Subsets and T Cell Function in Cartilage-Hair Hypoplasia**

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Cartilage hair hypoplasia is a rare autosomal recessive form of short-limbed dwarfism associated with a cellular immunodeficiency. In eight patients, the authors studied the presence of T cell subsets and *in vitro* T cell function in order to address the basis for the immunological disorder. Both the proliferative response to phytohaemagglutinin (PHA) and the PHA-induced IL2 production were 60% lower compared with controls (*P* = 0.007 and 0.005, respectively). The impaired proliferative response could not be restored by addition of IL-2. This result is in accordance with a decrease in the percentage of activated T cells expressing the p 55 subunit of the IL-2 receptor complex (CD25). The results define more precisely that T cells from cartilage hair hypoplasia patients are defective in the transition from the G0 to the G1 phase of the cell cycle. Furthermore, the data demonstrate that several CHH patients show a reduced proportion of CD45RA⁻ 'naive' T cells. However, the *in vitro* impairment of T cell function cannot solely be explained by imbalance between 'naive' and 'memory' T cells. Although CHH patients with a history of recurrent respiratory tract infections showed the most aberrant *in vitro* immune parameters, a clear relationship between clinical data and *in vitro* parameters could not be established for the whole patient group.

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**INTRODUCTION**

Cartilage-hair hypoplasia (CHH) is an autosomal recessive disorder comprising metaphyseal chondrodysplasia characterized by short-limbed dwarfism, fine sparse hair and an impaired cell mediated immunity. The clinical characteristics have originally been described in patients belonging to the Old Order Amish in the United States [1] and in Finns [2, 3]. The gene for CHH has been assigned to chromosome 9 [4], but has not been identified yet.

The impaired cell mediated immunity is indicated by lymphopenia, a decreased *in vitro* lymphocyte reactivity to mitogens, antigens and allogeneic cells [5-10] and by an increased susceptibility to infections in some patients [8, 10]. The humoral immunity was found to be intact [11]. Intrinsic defects in the proliferation of T cells, B cells and fibroblasts have been described previously [6]. T cell proliferation is dependent on a multiplicity of inter-related events such as the induction of interleukin-2 (IL-2) and its own receptor (IL-2-R) in the early G1 phase and the receptor for transferrin in the late G1 phase [12, 13]. Binding of IL2 to its own receptor is the actual signal which drives T cell entry into DNA synthesis. To test the hypothesis that T cell dysfunction in CHH patients is caused by certain defective steps in the transition of T cells from G0- phase into the G1- and the S-phase of the cell cycle, we investigated IL2 production in peripheral blood mononuclear cells (PBMC) and the induction of the transferrin-receptor (CD71) and the p55 subunit of the IL2-R (CD25) in CD4⁺ and CD8⁺ T cells. In addition to a T cell defect, the immunological disorder in CHH patients can also be the consequence of an imbalance in the 'naive' and 'memory' T cells. This has been described to occur in a number of diseases [14-16], but the proportions of 'naive' and 'memory' T cell subsets in patients with CHH have not yet been assessed. Therefore, we assessed the fractions of CD45RA⁺ ('naive') and CD45R0⁺ T ('memory') T cells.

It was shown that the PHA-induced IL2 production is impaired in CHH patients. The impaired proliferative response could not be restored by exogenous IL2. This result is in accordance with our finding that the expression of the p55 subunit of the IL2-R
following in vitro T cell activation is also reduced. In addition, several CHH patients show reduced proportions of CD45RA+ ‘naive’ T cells.

MATERIALS AND METHODS

Subjects. The patients were physically examined by one of the authors (CvdB, AH, CW). The diagnosis of CHH was based on the presence of disproportionate short stature because of metaphyseal chondrodysplasia, fair but slowly growing hair and a dysfunction of the cell-mediated immunity. Patients 1, 2, 3, 7, and 8 have been described previously [10].

The clinical findings of the eight patients are summarized in Table 1. Recurrent infections were defined according to Plebani et al. [17] as the occurrence of at least seven episodes of infection within 1 year, five or more episodes/year during 2 years, or at least three episodes/year during 3 years. Patients that fulfil these conditions are considered to have been immunodeficient during this period.

The control group for the patients consisted of healthy adult volunteers between 20 and 45 years of age. The results of mitogen-induced DNA-synthesis and IL2 production of children of the age of our patients and adults are not significantly different ([18, 19] and own unpublished observations).

Reagents. Biotinylated goat anti-mouse IgG was purchased from Oncogene Science (Manhasset, NY, USA). CD29 [4B4] was obtained from Coulter Electronics (Luton, UK) and tricolour-conjugated streptavidin from Caltuc (San Francisco, CA, USA). The MoAbs CD45RA (leu 18), CD45RO (UCHL1), CD25, CD71 and lineage specific MoAbs were obtained from Becton Dickinson (Mountain View, CA, USA). Phytomenagglutinin (PHA; HA15) was purchased from Becton Dickinson (Mountain View, CA, USA) and IL2 from Boehringer Mannheim (Mannheim, Germany).

Results

IL2 production1

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sib-pair</th>
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<tbody>
<tr>
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<tr>
<td>Sex and age in years</td>
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<td>M/12</td>
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<tr>
<td>Clinical features</td>
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<td>+</td>
</tr>
<tr>
<td>Recurrent respiratory tract infections in childhood</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Severe varicella infectiona</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.40</td>
<td>1.07</td>
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<tr>
<td>GH therapy for 2 years</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Results</td>
<td></td>
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<tr>
<td>Low proportion of CD45RA+ cells within the CD4+ subsetb</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Low proportion of CD45RA+ cells within the CD8+ subsetb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proliferative response to PHAc</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>IL2 productionc</td>
<td>141</td>
<td>3</td>
</tr>
</tbody>
</table>

a Normal (−) or severe (+) varicella infection is indicated.

b Below the 5th percentile of controls.

c As a percentage of normal mean values for the proliferative response and the IL2 production. The proliferative response for CHH patients ranged from 2626 to 13,748 cpm, and the IL2 production from 33 to 1464 pg/ml.

*nd = not determined.

Cells. Peripheral blood was drawn from patients and healthy adult donors by venupuncture, and PBMC were purified as described previously [20]. The blood from the patients was taken in a period when they were in good health.

Immunofluorescence. For two-colour flowcytometry, PBMC (0.5 × 10⁶) were labelled with a 1:10 dilution of fluorescein isothiocyanate (FITC)- and PE-labelled antibodies in minimal essential medium (MEM) containing 1% BSA and 0.02% NaN₃ (MEM/BSA/N₃). After 30 min incubation at 50°C, the cells were washed twice in MEM/BSA/N₃, at 50°C. T cell subsets were assessed by labelling with the following antibody combinations: anti-ab-FITC with anti-gd-PE; CD4-FITC with either CD45R0-PE or CD45RA-PE; and CD8-FITC with either CD45R0-PE or CD45RA-PE. Subsequently, cells were incubated with FITC-conjugated MoAbs against lineage-specific markers (CD4 or CD8) and PE-labelled CD45RO. Flow cytometric analysis was performed as described previously [21].

Cell cultures. To assay DNA synthesis, PBMC were cultured in the presence of 40 μg/ml PHA or 10 U/ml IL2 in RPMI (Gibco) containing 10% FCS (Gibco) as described earlier [20]. To determine IL2 production and the induction of activation markers (CD25 and CD71), cells were cultured with 40 μg/ml PHA in flat-bottomed microtiter plates at a concentration of 5 × 10⁵ lymphocytes/well. After 18 h the supernatant from 10 wells was pooled into two fractions and the IL2 concentration in the medium was determined using an ELISA system from Amersham International (Amersham, UK). The expression of activation markers (CD25 and CD71) on the cells was performed by flow cytometric analysis as described above.

Statistical analysis. Significant differences (P < 0.05) between patients and controls were calculated by using the Mann–Whitney U-test (nonpaired Wilcoxon test). Since the distributions were asymmetric for several T cell subsets, the arithmetic mean and SD cannot be used as measures for central tendency and population dispersion. For this reason
median and interquartile ranges (P25 and P75) were used as quantitative descriptors of cell population distribution. Since many observed immunological abnormalities varied widely between the patients, values for individual patients were compared with values for controls and considered as abnormal when they were below the 5th percentile (<P5) or above the 95th percentile (>P95) of the control group.

RESULTS

CD4+ and CD8+ T cell subsets in peripheral blood mononuclear cells

The percentages of various different lymphocyte subsets in Ficoll–Paque isolated PBMC of the patients and the controls are presented in Table 2. Since age-effects on the proportions of CD4+ and CD8+ T cell subsets are not significant for children older than 7 years of age [22, 23] the values for all patients, ranging in age from 7 to 37 years, were compared with the values for adult controls.

All but one patient showed a moderately reduced number of Ficoll–Paque-isolated PBMC. In six patients, the number was below the 5th percentile (<P5) value of controls (Table 2). The finding of reduced numbers of PBMC is in agreement with earlier documented lymphopenia in the patients [10]. Flow cytometric analysis also showed that no blast-like cell fraction was present. A reduced percentage of CD8+ cells (<P5) was found in four patients (pt. 2, 3, 4 and 8), whereas a reduced percentage of CD4+ T cells was observed in only one patient (Pt. 2). A reduced proportion of T cells with the αβ T cell receptor was found in patient 2, and T cells with the γδ T cell receptor were proportionally increased in pt. 2 and his twin sister pt. 3, but present in normal concentrations in the other patients tested (Table 2).

Naive and memory T cell subsets

Although the correlation is not complete, CD45RA and CD45R0 mark ‘naive’ and ‘memory’ T cells, respectively [24]. Flow cytometric analysis revealed that the proportion of CD45RA+ cells within the CD4+ subset of pts. 1, 2, 4 and 5 was below the P5 values for controls (Table 3). In these patients, the proportion of CD45R0+ cells was increased (as could be expected) because both isoforms of CD45 are mutually exclusive on most T cells. Since a large proportion of immature thymocytes are CD45RA-CD45R0+ [25, 26], we tested whether the increased percentage of CD45R0+ cells can be explained by the presence of immature thymocytes. However, immature double-positive (CD4+CD8+) cells were not found in the peripheral blood of any patient. An increased proportion of CD45RA+ cells was found in patients 3, 6 and 7 (>P95). However, it should be noted that the percentage of CD45RA+ cells within CD4+ cells in children from 7 to 17 years of age has been shown to be about 60% compared with about 40% in adults [27, 28]. This indicates that patient 6, because of his young age, has a normal percentage of naive CD4+ T cells and that patients 3 and 7 have increased percentages. Furthermore, the effect of age on naive CD4+ T cells implies that imbalance between CD45RA+ and CD45R0+ cells in the young patients 2 and 5 is even more pronounced, as appears in Table 3. The finding that there is an imbalance between the ‘naive’ and ‘memory’ subsets in most of the patients is supported by the results with the marker CD29, which is preferentially expressed on ‘memory’ T cells [29] (Table 3). With respect to ‘naive’ cells within the CD8+ subset patients 1 and 4, both adults, showed low expression of CD45RA (Table 3).

Notably, the lowest proportions of CD45RA+ cells in the CD8+ subset were found in the three patients that also showed a low proportion of CD45RA+ cells within the CD4+ subset (Table 3: patients 1, 2 and 4).

DNA synthesis and IL2 production

The mean in vitro proliferative response of CHH lymphocytes

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Table 2. Percentages of T cell subsets in CHH patients and controls

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<tr>
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<th>Pt. 1</th>
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<tr>
<td>PBMCb</td>
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<td>710i</td>
<td>1550</td>
<td>410i</td>
<td>570i</td>
<td>630i</td>
<td>760i</td>
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<td>CD4+</td>
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<td>8.8i</td>
<td>37</td>
<td>35</td>
<td>28</td>
<td>40</td>
<td>49</td>
<td>44</td>
<td>37</td>
<td>70</td>
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<tr>
<td>CD8+</td>
<td>22</td>
<td>12i</td>
<td>15i</td>
<td>6.8i</td>
<td>20</td>
<td>20</td>
<td>11i</td>
<td>9.0i</td>
<td>14</td>
<td>21</td>
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<tr>
<td>CD4/CD8</td>
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<td>0.7i</td>
<td>2.5</td>
<td>5.2i</td>
<td>1.4</td>
<td>2.0</td>
<td>4.3i</td>
<td>4.9i</td>
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<tr>
<td>TeR αβc</td>
<td>55</td>
<td>18i</td>
<td>44</td>
<td>nda</td>
<td>nd</td>
<td>nd</td>
<td>64</td>
<td>54</td>
<td>54</td>
<td>61</td>
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<tr>
<td>TeR γδc</td>
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<td>17i</td>
<td>11i</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>2.3</td>
<td>2.8</td>
<td>3.5</td>
<td>2</td>
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</table>

a Data are expressed as medians with ranges from the 25th to the 75th percentiles in parenthesis. n = 32 for controls.

b Absolute number of cells in Ficoll-paque-isolated PBMC per mm³.

c TeR = T cell receptor.

i Proportion of cells above the 95th percentile of controls.

† Proportion of cells below the 5th percentile of controls.

Psignificantly different from controls (P < 0.05).

nd = not determined.
Table 3. CD29 and CD45 isoform expression on CD4⁺ and CD8⁺ cells from CHH patients and controls

<table>
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<th>Pt. 5</th>
<th>Pt. 6</th>
<th>Pt. 7</th>
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<tr>
<td>CD4 %CD45RA⁺</td>
<td>91</td>
<td>161</td>
<td>83</td>
<td>31</td>
<td>261</td>
<td>62</td>
<td>63</td>
<td>47</td>
<td>37 (14–62)</td>
<td>43 (30–60)ᵇ</td>
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<tr>
<td>CD4 %CD45R0⁺</td>
<td>83</td>
<td>77</td>
<td>26</td>
<td>92</td>
<td>69</td>
<td>41</td>
<td>28</td>
<td>47</td>
<td>58 (38–78)</td>
<td>45 (35–65)ᵇ</td>
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<tr>
<td>CD4 %CD29⁺</td>
<td>62</td>
<td>68</td>
<td>25</td>
<td>nd</td>
<td>58</td>
<td>34</td>
<td>12</td>
<td>17</td>
<td>36 (17–42)</td>
<td>26 (22–36)ᵇ</td>
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<tr>
<td>CD8 %CD45RA⁺</td>
<td>29</td>
<td>39</td>
<td>68</td>
<td>28</td>
<td>66</td>
<td>77</td>
<td>73</td>
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<td>63 (36–69)</td>
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<td>17</td>
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<td>36 (17–42)</td>
<td>26 (22–36)ᵇ</td>
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<tr>
<td>CD8 %CD29⁺</td>
<td>82</td>
<td>77</td>
<td>46</td>
<td>nd</td>
<td>51</td>
<td>30</td>
<td>41</td>
<td>37</td>
<td>51 (35–58)</td>
<td>39 (31–47)ᵇ</td>
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</table>

ᵇ The percentage of CD45RA⁺, CD45R0⁺ and CD29⁺ cells were determined on PBMC gated for either CD4 or CD8.
ᵇ Data are expressed as medians with ranges from the 25th to the 75th percentiles in parenthesis. n = 15 for controls.
ᵇ Values obtained by others for the %CD45RA on CD4⁺ T cells of 7–17-years-old children and adults are 61 (55–67) and 40 (32–49), respectively (29).
ᵇ Proportion of cells above the 95th percentile of controls.
ᵇ Proportion of cells below the 5th percentile of controls.
ᵇ nd = not determined.

to PHA was significantly reduced compared with controls (P < 0.026; Fig. 1). The IL2 production in PBMC from CHH patients is significantly lower compared with controls (P = 0.005; Fig. 1), and there is no relation between the IL2 production in the first 18 h after activation and the expression of DNA synthesis (see also Table 1). The impaired response could not be restored by exogenous IL2 (Fig. 1).

To assess to what extent reduced T cell proportions in PBMC are responsible for the impaired in vitro proliferation and IL2 production, we determined the percentage of T cells (CD3⁺) in PBMC of each patient (Fig. 1). It appears that there is no relation between the T cell proportion and IL2 production, and that the IL2 production is much more reduced than the proportion of T cells. On the other hand, the lowest proliferation is found in patients with the smallest proportion of T cells (patients 1, 2, 3 and 5). However, the proliferative response is more reduced than the percentage of T cells (Fig. 1).

To address the responsiveness of resting (unstimulated) PBMC from CHH patients to IL2, we tested whether IL2 could induce DNA synthesis. It has been established that a small
fraction of PBMC express receptors for IL2 and will enter the S-phase in response to IL2 [30]. The induction of DNA synthesis by IL2 showed no differences between patients and controls. Furthermore, the patients that responded poorly to PHA (patients 1, 2 and 5) exhibited moderate responses to IL2 (data not shown).

**Expression of IL2 receptors (CD25) and transferrin receptors by PHA**

To address the events which occur during the transition from the G0 to the G1 phase of the cell cycle, we determined the expression of the p55 subunit (CD25) of the IL2-R complex, and the expression of transferrin receptors (CD71) at 18 and 40 h after activation with PHA. Figure 2A reveals that the expression of CD71 in CD4+ T cells from CHH patients was normal. The proportion of CD25+ cells 18 h after stimulation was slightly lower in CHH patients compared to controls (Fig. 2B). After 40 h, the mean expression of CD25 was significantly less on CD4+ cells from CHH patients (P = 0.05). Furthermore, in patients 1, 2, 4, 5 and 6 the proportion of CD25+ cells decreased between 18 and 40 h after activation (Fig. 2B). This phenomenon was never observed in activated cells from controls.

Similar to that observed for CD4+ cells, the expression of CD71 on CD8+ cells at 18 h was not different from the controls (Fig. 2C). However, at 40 h, we found a relatively low proportion of CD71+ cells in three patients (patients 1, 2 and 4). The mean percentage of CD25+ within the CD8+ population from CHH patients was not significantly different from controls at 18 h after stimulation (Fig. 2D), but at 40 h the proportion of CD25+ cells was significantly lower compared with controls (P = 0.015). The proportion of CD25+ cells was relatively low in one patient.

Fig. 2. Induction of transferrin receptors (CD71) and p55 subunits of the IL2 receptor (CD25) in CD4+ and CD8+ cells. PBMC were cultured in the presence of PHA. After 18 and 40 h, the percentage of CD25+ and CD71+ cells within the CD4+ and CD8+ subsets was determined using two-colour flow cytometry. The figures represent the percentage of CD4 or CD8 gated cells that expressed CD25 or CD71. (A) CD71 expression on CD4+ cells; (B) CD25 expression on CD4+ cells; (C) CD71 expression on CD8+ cells; (D) CD25 expression on CD8+ cells. Median values and ranges from 25th to 75th percentiles are represented as horizontal bars and vertical bars, respectively. *P < 0.05 compared with controls.

at 18 h after stimulation and in three patients at 40 h after stimulation. Notably, despite a relatively normal expression of CD25 in patients 1 and 4, the percentage CD25+ cells decreased between 18 and 40 h after stimulation.

A DISCUSSION

The T lymphocyte defect in CHH patients is as yet not fully understood. Early work from Pierce & Polmar [5, 6] clearly showed that an intrinsic T cell defect in the proliferative capacity of lymphocytes does exist. We found that all but one patient showed an impaired production of the autocrine T cell growth factor IL2 as measured after 18 h of activation (Fig. 1). However, there is no relation between the level of IL2 production, and the proliferative response and addition of IL2 to the culture medium did not restore this response (Fig. 1). This result implies that a reduced production of IL2 is not the only cause of the impaired proliferative response, which is in accordance with our observation that the proportion of T cells that express the p55 subunit of the IL2 receptor (CD25) is reduced in PHA activated T cells. Since the IL2 induced DNA synthesis is not impaired in non-stimulated PBMC from CHH patients (data not shown), we conclude that there is no defect in IL2 receptor function and that CHH lymphocytes have a normal capability to enter the S phase. Taken together, our data confirm the assumption of Pierce & Polmar [5, 6] that there is a partial cell cycle defect in the transition from the G0 to the G1 phase. This hypothesis is in accordance with the observation that one CHH patient with combined immunodeficiency was found to have a defective expression of early activation genes like c-myc, IL2Ra, IL2 and IFN-γ as assessed by mRNA measurements [31]. Notably, in several patients the percentage of CD25+ cells was lower after 40 h compared to 18 h. Therefore, we have to take into account the possibility that the cell cycle defect affects progression through the G1 phase instead of, or in addition to, the transition from the G0 to the G1 phase. The decrease in CD25+ cells between 18 and 40 h suggests that certain cells will not enter the S phase, which explains the impaired proliferative response. However, we cannot exclude the possibility that the peak response of all cells is shifted to a later time point.

Both CD4+ and CD8+ peripheral T cells can be divided into CD45RA+ and CD45R0+ subsets. Although there is no strict correlation, CD45RA is mainly expressed on 'naive' T cells which are not yet exposed and responsive to antigen, whereas CD45R0 marks the 'memory' T cell stage [24, 32]. In addition, CD29 has been shown to be mainly expressed on 'memory' T cells [24]. 'Naive' and 'memory' T cells differ in responsiveness to mitogens and antigens, and exert different immunological functions. Importantly, the proliferative response to PHA and concomitant IL2 production is more pronounced in CD45RA+ 'naive' T cells compared with the CD45R0+ 'memory' cells [24, 33]. Therefore, impaired T cell function as measured by in vitro assays using PBMC might be related to the proportion of CD45RA+ cells [16]. Using these maturation stage dependent markers, we found that the patients with the smallest proportion of CD45RA+ cells within the CD4+ population (<5%) had indeed the lowest proliferative response to PHA (Table 1). However, there was no relation between the presence of naive and memory T cell subsets and IL2 production. While two patients with a low proportion of CD45RA+ T cells show the lowest IL2 production (patients 2 and 4; Table 3 & Fig. 1), two other patients with a relatively low IL2 production (31 and 35% of the normal mean values) have normal percentages of CD4+ and CD8+ cells in PBMC and relatively high proportions of CD45RA+ cells within these subsets (patients 6 and 7). In addition, lymphocytes of patient 1 produced a high amount of IL2, in spite of a low percentage of CD45RA+ T cells (Table 3 & Fig. 1). These results indicate that in CHH patients a reduced proportion of 'naive' T cells is not systematically related to a decreased IL2 production. We conclude that the in vitro T cell defects cannot be explained solely by the imbalance of 'naive' and 'memory' cells. On the other hand, patients with recurrent respiratory tract infections in childhood had the smallest proportions of 'naive' (CD4+ and CD8+) T cells (Tables 1 & 3). Instead of a primary defect, this phenomenon might be a secondary effect due to an increased number of infections during childhood. In this respect it should be noted that two adult patients that no longer suffer from recurrent infections still show low proportions of 'naive' T cells (Table 1). Our finding that the majority of patients with CHH in our study show a reduced fraction of 'naive' T cells stresses the importance of addressing these subsets in further clinical studies.

With respect to the clinical features we conclude that none of the immunological abnormalities we described is related to GH hormone treatment instituted in four patients (Table 1). This result is in agreement with earlier observations which reveal that GH does not have, or hardly has, any effect on the immune function in children [34, 35]. In a separate study [36] we determined type I insulin growth factor (IGF) receptor expression on T cell subsets. In healthy individuals both 'naive' and 'memory' T cells contain type I IGF receptor positive and negative cells [37]. Interestingly, CHH patients with a history of recurrent infections also showed the lowest percentage of type I IGF receptor bearing 'naive' T cells both in the CD4+ and CD8+ subset ([36], data not shown). There was no strict correlation between the PHA-induced IL2 production and occurrence of recurrent infections in childhood (Table 1, Fig. 1). On the other hand, the percentage of CD25+ T cells at 40 h after PHA-activation was markedly lower in T cells from patients with a history of recurrent infections than in the other patients and normal controls (Fig. 2). This is also in agreement with our finding that the PHA-induced proliferative capacity is most strongly affected in patients with a history of infections. Thus, the proliferative defect in T cells from CHH patients is, at least in part, caused by a reduced capability to express the products of early activation genes like IL2, the IL2 receptor and the transferrin receptor. However, the nature of the defect that affects T cells of CHH patients is as yet unclear. In addition, the relationship between dysfunctional in vitro parameters and clinical data, although present in a
REFERENCES


