

Effect of growth hormone therapy on the proinflammatory cytokine profile in growth hormone-deficient children

Sara Pagani, Cristina Meazza, Paola Travaglini, Antonia Moretta, Mauro Bozzola

Dipartimento di Scienze Pediatriche, Università degli Studi di Pavia, piazzale C. Golgi 2, 27100 Pavia, Italy

Correspondence : M. Bozzola
<mauro.bozzola@unipv.it>

ABSTRACT. The aim of the present study was to establish whether growth hormone (GH) treatment *in vivo* affects pro-inflammatory cytokine production by resting or *in vitro*, activated, cultured, peripheral blood mononuclear cells (PBMC) from children with complete growth hormone deficiency (GHD). We evaluated 11, pre-pubertal children (6 males and 5 females) with GHD, aged between 6 and 14 years, and 9, age- and sex-matched healthy subjects were studied as controls (CTRLs). Freshly isolated PBMC were cultured for 4 or 24 h in X-VIVO medium in the presence or absence of 0.01 µg/mL lipopolysaccharide for the determination of TNF- α and IL-6 production; alternatively, cells were incubated 24 h in X-VIVO medium with or without 25 µg/mL Concanavalin A for IFN- γ production. Cytokines were measured in the cell supernatants by enzyme-linked immunosorbent assay kits. The results of the present study provide evidence that spontaneous and/or mitogen-induced, *in vitro* PBMC production of pro-inflammatory cytokines is lower in GHD children than in healthy, age-matched individuals ($p < 0.05$ by the Mann-Whitney U-test). After 3 months of GH therapy, cytokine production was significantly ($p < 0.05$ by the Wilcoxon test) increased, but was still lower than in healthy controls. It is reasonable to speculate that severe GH deficiency can cause alterations in the pro-inflammatory cytokine-induced immune response in humans, and that GH treatment can ameliorate this important immunological function.

Keywords: proinflammatory cytokines, peripheral blood mononuclear cells, growth hormone deficiency, immune system

Recent reports suggest a complex interplay between the neuroendocrine and the immune systems. Immune cells release soluble factors that affect neuroendocrine mechanisms, and conversely, neuroendocrine hormones can affect immune function [1]. A communication network between cells of the neuroendocrine and those of the immune system seems to occur by means of the secretion of soluble factors. Cells of the immune system are known to secrete and/or have receptors for several hormones [2]. In particular, growth hormone (GH) and the insulin-like growth factors (IGFs) play an important role in immune system function, by affecting both cell-mediated and humoral responses [3]. For instance, experimental data demonstrated that GH can stimulate lymphocyte proliferation, especially of B-lymphocytes, possibly *via* IGF-I, and elicits immunoglobulin synthesis [4-7]. Considerable evidence demonstrates that IGF-I, endogenously produced or exogenously added, affects *in vitro* immune cell replication and function and stimulates the proliferation of T cells [4, 8]. In addition, IGF-I increases mitogen-induced IL-2 production [9], which might be responsible for the effect on proliferation. Moreover, some cases of primary, humoral immunodeficiency syndrome associated with GH deficiencies have been reported, even though it is still

controversial whether the two disorders should be considered a definite disease entity [10-12].

Cytokines are a large family of protein mediators, including interleukins (ILs), colony-stimulating factors (CSFs), interferons (IFNs), tumor necrosis factors (TNFs) and growth factors, which are produced by a wide variety of cells. Cytokines have been implicated as regulators of the development of hematopoietic cells as they may influence survival, proliferation, differentiation and homeostasis of lymphoid cells [13]. They play a major role in the initiation and regulation of immune and inflammatory responses. Activation of pro-inflammatory cytokine production by monocytes is a crucial step in host defense against pathogens, but if embroidered or inappropriate, may also result in severe tissue injury and septic shock [14].

A number of *in vitro* studies has demonstrated a modulating effect of human GH on cytokine secretion. In particular, Mustafa *et al.* showed that hGH is able to enhance, in a dose-dependent manner, interferon- γ (IFN- γ) production by human peripheral blood mononuclear cells (PBMC) from healthy subjects cultured with different hGH concentrations [15]. Furthermore, hGH enhances IL-1 α , TNF- α and IL-6 production by lipopolysaccharide (LPS)-activated monocytes in whole blood [14]. It was also

demonstrated that monocyte production of IL-6 and TNF- α is increased in patients with complete GHD but partially normalizes after GH replacement therapy [16]. Even though much information is available on the influence of GH on the immune system, the relationship between the GH-IGF-I axis and humoral function through autocrine, paracrine or endocrine pathways still requires elucidation and the clinical importance of this interaction has not been definitely demonstrated.

The aim of the present study was to obtain further insight into the interaction between GH and the immune response and, in particular, to establish whether GH treatment *in vivo* affects pro-inflammatory cytokine production from resting or *in vitro*, activated, cultured PBMC from children with complete growth hormone deficiency (GHD).

PATIENTS AND METHODS

Patients

We evaluated 11, pre-pubertal children (6 males and 5 females) with complete growth hormone deficiency (GHD), documented by serum GH peak levels less than 5 ng/mL, in response to at least two pharmacological stimuli. Mean \pm SD chronological age was 11.3 ± 2.62 years, mean \pm SD bone age was 8.5 ± 2.8 years, height was -1.53 ± 1.02 SDS, and body mass index was 0.18 ± 1.58 SDS.

Nine, age- and sex-matched, healthy subjects, taking part in a screening program for hyperlipidaemia at school, were studied as controls (CTRLs).

Blood samples were collected from the patients between 08.00 hours and 09.00 hours before the 1st GH subcutaneous injection (0.1 IU/kg/day for 6 days/week) and then after three months of GH therapy. Informed consent was obtained from the children's parents.

Measurement of cytokine levels

PBMC were isolated by density centrifugation using Ficoll/Histopaque (Lymphoprep; Nicomed, Oslo, Norway) and resuspended in serum-free X-VIVO medium (Biowhittaker, Walkersville MD, USA) supplemented with 0.05 mg/mL gentamicin (Gibco, Invitrogen Corporation, Paisley, UK). For the determination of TNF- α and IL-6 production, freshly isolated PBMC were cultured respectively for 4 or 24 h in X-VIVO medium in the presence or absence of 0.01 μ g/mL LPS (from *E. coli* 055:B5, Sigma-Aldrich, ST. Louis MO, USA); for IFN- γ production, cells were incubated for 24 h in X-VIVO medium with or without 25 μ g/mL Concanavalin A (ConA, Pharmacia, Upsala, Sweden). Cytokines were measured in the cell supernatants by enzyme-linked immunosorbent assay kits (DuoSet R&D Systems, Minneapolis MN, USA). The minimum detectable concentrations from these assays were 7.0 pg/mL for TNF- α , 2.34 pg/mL for IL-6 and 7.81 pg/mL for IFN- γ . The intra- and inter-assay coefficients of variation were less than 7%.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical differences between patients before therapy and the controls were

determined using the Mann-Whitney U test; the non-parametric Wilcoxon test for paired samples was used to compare cytokine levels in patients before and after 3 months of GH treatment; $p < 0.05$ was considered statistically significant.

RESULTS

As shown in *figure 1*, TNF- α production by LPS-stimulated PBMC was significantly lower ($p=0.006$) in GHD children before GH therapy (45 ± 23.8 pg/mL) as compared with healthy CTRLs (229.0 ± 57.5 pg/mL). After 3 months of GH therapy, we found a significantly increased capacity for LPS-stimulated TNF- α production (110.0 ± 71.2 pg/mL), when compared with that obtained before treatment ($p < 0.01$). However, values for TNF- α production 3 months after GH therapy were still significantly lower ($p=0.015$) than those obtained from healthy CTRLs. Spontaneous *in vitro* TNF- α production was lower in GHD patients before therapy (3.6 ± 2.2 pg/mL) than in CTRLs (5.4 ± 0.3 pg/mL) and was considerably augmented after 3 months of GH-therapy (10.6 ± 3.3 pg/mL), but the differences did not reach statistical significance.

LPS-stimulated PBMC from patients, before and after GH treatment, showed an IL-6 production comparable to that of healthy CTRLs (*figure 2*), while spontaneous *in vitro* production of IL-6 by PBMC from GHD children obtained before GH-therapy (48 ± 1.1 pg/mL) was significantly lower ($p=0.0006$) than in healthy CTRLs (1644.0 ± 761.4 pg/mL). After 3 months of GH-therapy, spontaneous IL-6 production was significantly increased (305.9 ± 112.8 pg/mL) when compared to that measured before therapy ($p=0.009$), but was still lower than that of healthy CTRLs ($p=0.0006$).

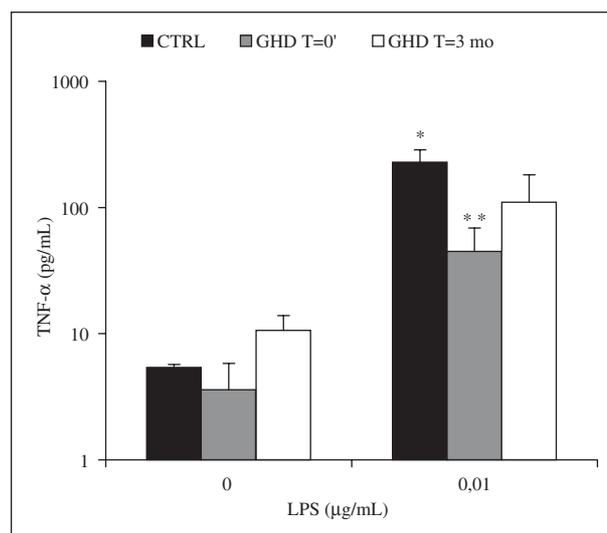


Figure 1

Effect of GH therapy on *in vitro* production of TNF- α by unstimulated and LPS-stimulated PBMC of GHD children before (shaded bars) and after 3 months of treatment (open bars) in comparison with healthy controls (CTRL, solid bars).

Results are represented as mean \pm SEM.

* T=0 versus corresponding CTRLs $p < 0.05$ (Mann-Whitney U test).

** 3 months versus corresponding T=0' $p < 0.05$ (Wilcoxon test).

° 3 months versus corresponding CTRLs $p < 0.05$ (Mann-Whitney U test).

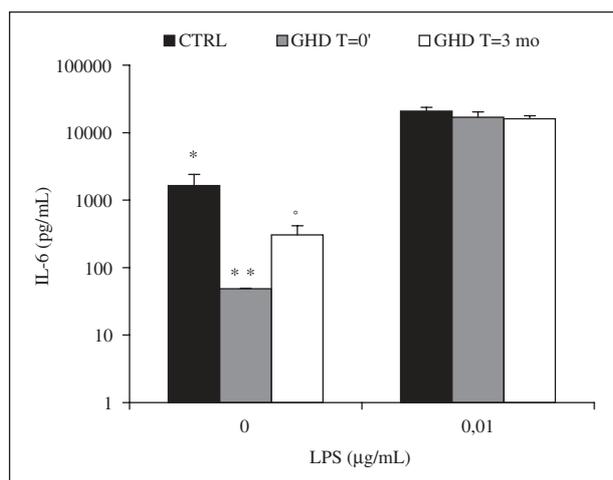


Figure 2

Effect of GH therapy on *in vitro* production of IL-6 by unstimulated and LPS-stimulated PBMC of GHD children before (shaded bars) and after 3 months of treatment (open bars) in comparison with healthy controls (CTRL, solid bars).

Results are represented as mean + SEM.

* T=0 versus corresponding controls $p < 0.05$ (Mann-Whitney U test).

** 3 months versus corresponding T=0 $p < 0.05$ (Wilcoxon test).

° 3 months versus corresponding CTRLs $p < 0.05$ (Mann-Whitney U test).

PBMC from GHD children before therapy, when stimulated with Con A, showed a significantly reduced production of IFN- γ compared to that of healthy controls (GHD: 325.17 ± 71.4 pg/mL and CTRL: 842.5 ± 194.3 pg/mL; $p = 0.03$) (figure 3). After 3 months of GH treatment, ConA-stimulated production (493.23 ± 106.5 pg/mL) of IFN- γ was significantly increased when compared to that obtained before treatment ($p < 0.04$), but was still lower than that of healthy CTRLs. Spontaneous IFN- γ production was similar in patients, before and after GH-therapy, and in healthy CTRLs.

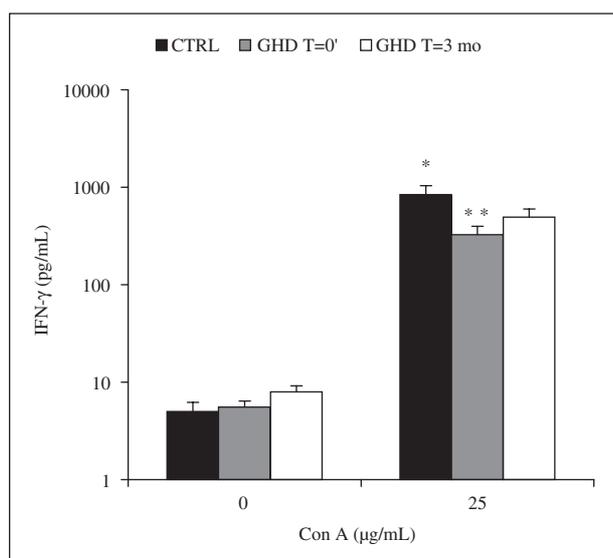


Figure 3

Effect of GH therapy on *in vitro* production of IFN- γ by unstimulated and Con A-stimulated PBMC of GHD children before (shaded bars) and after 3 months of treatment (open bars) in comparison with healthy controls (CTRL, solid bars).

Results are represented as mean + SEM.

* T=0 versus corresponding controls $p < 0.05$ (Mann-Whitney U test).

** 3 months versus corresponding T=0 $p < 0.05$ (Wilcoxon test).

DISCUSSION

It has long been postulated that immune and neuroendocrine systems interact at various levels for modulating inflammatory and immune responses. In particular, hormones such as prolactin and GH are thought to act as immunostimulators [17]. Furthermore, the GH receptor (GHR), a member of the type 1 cytokine receptor family (IL-2R, IL-4R and IL-6R), is expressed on different lymphocyte subpopulations [3, 18], suggesting a local mechanism of action of this hormone in addition to the traditional endocrine mode of action. Many indirect GH effects can be mediated through IGF-I induction [3] as cells of the immune system such as T, B-lymphocytes and macrophages express functional IGF-I receptors [19, 20]. IGF-I is also produced by immune cells [21], so that its effect on immune responses may be secondary to autocrine or paracrine mechanisms.

The results of the present study provide evidence that spontaneous and/or mitogen-induced *in vitro* PBMC production of pro-inflammatory cytokines is lower in GHD children than in healthy, age-matched individuals, and 3 months of GH-therapy is able to ameliorate this important immunological function.

Several experimental studies have demonstrated that GH is able to modulate cytokine responses, even though conflicting data have been reported. Edwards *et al.* showed a reduction in macrophage TNF- α production after *in vitro* stimulation with LPS in hypophysectomized rats [22]. Other *in vitro* studies have demonstrated that incubation of PBMC with different concentrations of hGH significantly increases the number of interferon- γ -secreting cells, as well as the concentration of IFN- γ [15]. On the other hand, the THP-1 cell line, engineered to constitutively produce human growth hormone, secretes depressed amounts of TNF- α in response to challenge with LPS [23].

Conversely, GH therapy does not seem to affect serum IL-4 levels. In fact, our previous results showed comparable serum IL-4 levels in GHD children before and after GH therapy, and healthy controls (Pagani S. *et al.*, in press).

IL-4 is known to promote immune cell differentiation toward the Th2 phenotype and transform naïve Th cells into IL-4-producing T cells; Th2 cytokines promote Th2 activities and inhibit Th1 activities, and *vice versa*. Takagi *et al.* demonstrated that in burned mice, in which there is a conversion from Th1 cells to Th2 cells, rhGH administration leads to a Th1-dominant response, supporting the role of GH in modulating the release of type 1 cytokines [24]. The *in vivo* relevance of *in vitro* results has yet to be demonstrated because the clinical significance of the relationship between GH and cytokine production in humans is debatable, as clinical signs of immune dysfunction have been associated with severe GH deficiency only in a few patients [10-12].

Our results show that IFN- γ production by ConA-stimulated PBMC is significantly reduced in GHD patients compared with production in healthy individuals, and that the *ex vivo* synthesis of this cytokine significantly increases after three months of GH therapy. These results are in keeping with previous observations demonstrating an enhancing effect of GH on the cytotoxic activity mediated by natural killer (NK) lymphocytes, a cell subset known to be an important source of IFN- γ and to mediate MHC unrestricted killing of tumors and virally-infected cells

[25-27]. Furthermore, aged and hypophysectomized mice have been demonstrated to have a deficiency in NK activity and administration of GH reverses this defect [28]. Moreover cytokines such as TNF- α and IL-12 stimulate NK cells to secrete IFN- γ . NK cell-derived IFN- γ then stimulates macrophages to increase the production of TNF- α and IL-12, which in turn leads to more IFN- γ production by NK cells. This reciprocal stimulation forms a positive amplification loop that results in the rapid generation of substantial quantities of IFN- γ and a large number of activated macrophages. Interestingly, in this study we showed increased amounts of TNF- α in LPS-stimulated PBMC from GHD patients after three months of GH-therapy, with respect to production before the treatment. Increased levels of TNF- α could contribute to the increased production of IFN- γ in the supernatant of stimulated patients' PBMC.

We also found a significant increase in spontaneous IL-6 production in patients after three months of GH treatment compared to the production before the therapy. These results are in line with our unpublished data showing a significant increase in serum IL-6 levels, six hours after GH injection, in GHD children.

Our present data suggesting the influence of GH therapy on the production of pro-inflammatory cytokines are in keeping with previously published data demonstrating a significant increase in serum TNF- α and IL-1 β concentrations soon after GH injection, compared to basal levels, and a decrease in their levels 24 hours later, when serum GH values fell [29]. Furthermore, our previous study demonstrated that in non-GH-deficient, short children, serum levels of TNF- α , IL-1, IL-2, IL-12 and IFN- γ significantly increased 4 hours after GH injection, even if the basal values were comparable with those of healthy controls [30].

Our results are in contrast with data shown by Serri *et al.* [16]: we suppose that cytokine production may be different between adults and children.

In fact, adult patients with multiple pituitary hormone deficiency have increased cardiovascular risk factors, including altered body composition, with increased body fat and abnormal levels of serum lipids and lipoproteins, both of which were improved by GH replacement therapy. Evidence has been provided that immune mechanism play an important role in atherogenesis: in particular monocytes seem to contribute to the early development of atherosclerosis and produce proatherogenic cytokines such as IL-6 and TNF- α . For this reason, GHD adults show IL-6 and TNF- α levels higher than healthy controls [16].

In conclusion, it is reasonable to speculate that severe GH deficiency can cause impairment in the pro-inflammatory cytokine-induced immune response in humans, and that this defect can, at least partially, be corrected by GH-therapy. Nevertheless, as the impairment of *in vitro* cytokine production observed before GH-therapy was not correlated with immune-mediated clinical dysfunction in our patients, it is conceivable to hypothesise that, as suggested by Clark *et al.* [3], in humans, GH synthesized from the immune system is able to compensate the pituitary GH deficiency. In fact, experimental evidence demonstrated that the regulation of local GH in the immune system may be different from that in the pituitary, and so we suppose that GH is produced normally in lymphocytes [3].

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