LONGITUDINAL BEHAVIOUR OF AUTOIMMUNE GROWTH HORMONE DEFICIENCY: FROM CHILDHOOD TO TRANSITION AGE

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ABSTRACT

CONTEXT: Some cases of apparently idiopathic GH deficiency (GHD) may be caused by pituitary autoimmunity.

OBJECTIVE: To study the variations from childhood to transition age of pituitary function and pituitary antibodies (APA) in patients with apparently idiopathic GHD.

DESIGN: We conducted a longitudinal study.

PATIENTS AND METHODS: Pituitary function and APA detection by immunofluorescence were investigated in 24 childhood patients with isolated GHD before starting recombinant GH therapy and after the stopping of this therapy in transition age. Sera of patients positive for APA were processed by double immunofluorescence to identify their pituitary target.

RESULTS: At diagnosis, 16 out of 24 patients were APA positive targeting only somatotrophs GH-secreting cells (group 1), while the remaining eight APA negative (group 2). When retested off therapy, 12 out of 16 patients in group 1 persisted APA positive, while the remaining four became negative with recovery of pituitary function. All patients in group 2 persisted APA negative but still showing GHD. Among the 12 patients persisting APA positive, eight with confirmed GHD showed APA still targeting somatotrophs, whereas four showed APA targeting only gonadotrophs, associated with isolated hypogonadotropic hypogonadism (HH).

CONCLUSIONS: Patients with APA at middle but not at high titer in childhood age may show a remission of autoimmune GHD in childhood, after GH replacement therapy. Since pituitary antibodies may shift their target in transition period, an early characterization of APA by double immunofluorescence is advisable in APA positive GHD patients showing delayed puberty, to allow an early diagnosis and an appropriate therapy, thus preventing the progression toward HH.
INTRODUCTION

For a long time, on the basis of the detection of some organ-specific antibodies, many endocrine diseases, previously considered as idiopathic, have been recognized as autoimmune (1-6). In previous studies it has been reported that pituitary antibodies (APA) at high titers may be present in some patients with apparently idiopathic selective hypopituitarism suggesting an autoimmune pituitary involvement in these cases (7-9). However, since pituitary gland contains several different hormone-secreting cells, the four-layer double immunofluorescence may be considered an appropriate method to identify the secreting cells targeted by APA. By using this method we were able to identify selectively the type of pituitary hormone-secreting cells targeted by APA in adults and in children with isolated idiopathic GH deficiency (GHD), in patients with idiopathic hypogonadotropic hypogonadism (HH) and in those with isolated ACTH-deficiency (10-13). In particular, specific staining by double immunofluorescence either of somatotrophs or gonadotrophs or ACTH-secreting cells alone, characterized respectively isolated GHD, isolated HH or isolated ACTH-deficiency.

So far, possible changes of anterior pituitary function and APA in patients with isolated autoimmune hypopituitarism without imaging alterations of hypothalamic-pituitary region, have been observed only in patients progressing from subclinical to clinical phase (14). Instead, the possible time-related variations of APA and of anterior pituitary function before and after replacement therapy in patients in clinical phase of isolated autoimmune hypopituitarism and in particular in those with autoimmune GHD diagnosed and treated with replacement recombinant GH (rGH) therapy since childhood, have so far never been investigated.

Prompted by these observations, this longitudinal study was aimed at investigating in patients with childhood onset of autoimmune GHD, treated with rGH, possible time-related variations of pituitary antibodies and anterior pituitary function from childhood to transitional age period.

PATIENTS AND METHODS
Patients

We enrolled for this study 24 pre-pubertal patients with childhood onset GHD (14 males and 10 females, age range at diagnosis 7-9.1 years) among 36 patients diagnosed from 2002 to 2005 at the Endocrinology and Metabolism Unit of the University Hospital of the Second University of Naples as having idiopathic GHD. The diagnosis of idiopathic isolated GHD was established on the basis of the following criteria: short stature (age and sex adjusted height < 2 SD score (SDS), reduced growth velocity < 25th centile, delayed skeletal development and blunted GH response < 8 µg/L to two pharmacologic stimulation tests), as previously described (11). Magnetic resonance imaging (MRI) of the hypothalamic pituitary region and basal and dynamic secretion of other pituitary hormones were normal in all patients. All 24 patients, after the diagnosis and the basal assessment, started replacement rGH therapy, which was continued for about 8 years until the transition period (at initial dose of 0.030±10 mg/Kg/die). The period of transition was arbitrarily defined as period of late adolescence in which GH treatment is usually stopped for the achievement of adult stature. Twelve months after the interruption of rGH therapy, a complete reevaluation of pituitary function was performed until February 2015. Moreover, APA, already evaluated in all patients at the enrolment, were re-evaluated after rGH interruption in transition period and MRI of hypothalamic-pituitary region, already performed at diagnosis, was repeated after the stopping of therapy. Patients and their parents gave informed written consent to participate to the study, which was approved by the Institutional Review Board.

Methods

APA evaluation

APA were detected by simple indirect immunofluorescence method on cryostat sections of young baboon pituitary gland supplied by Halifax spa (Polverara, Pordenone, Italy), as previously
described (7). In particular, fluorescein isothiocyanate (FITC) conjugated goat antihuman Ig was used to detect the presence of APA; they were considered positive starting at dilution of 1:8. In our laboratory APA present at titer ranging from 1:16 to 1:32 are considered positive at middle titer; those positive > 1:32 are considered to be at high titer.

APA were also evaluated both in sera of 30 sex/age-matched healthy subjects followed from childhood age to transition age. Moreover, sera of APA positive patients and 4 control sera negative for these antibodies were retested by a four layer double immunofluorescence to characterize the pituitary cells targeted by APA (11). In particular, after the first immunostaining step, using fluorescein isothiocyanate (FITC) conjugated goat antihuman Ig, a second immunostaining step using rabbit antisera anti ACTH, GH, TSH, PRL LH, FSH separately (Histo2line Srl, Milano), followed by rodhamine anti rabbit Ig, was performed. The close correspondence between the cells colored in green in the first step and those colored in red in the second step, after the treatment with the antisera anti each pituitary hormone, separately, followed by rodhamine anti rabbit Ig, indicates what pituitary-secreting cells are targeted by APA.

**Anterior pituitary function**

Basal anterior pituitary hormones (ACTH, FSH, LH, TSH, GH and PRL) and their respective target organ hormones (cortisol, gonadal hormones, FT4, FT3, IGF-1) and dynamic evaluation were performed as previously described (14). In particular, the diagnosis of GHD in transition phase after the stopping of GH therapy was defined by growth velocity less than 2 cm/year, low IGF-1 levels below 2 SD of normal range and a GH peak < 11 µg/L to GHRH +/ arginine in patients with body mass index (BMI) ranging from 20-25 kg/m², GH peak <8 µg/L in those with BMI from 25 to 30 kg/m² and GH peak <4 µg/L in those with BMI > 30 kg/m² (15). The diagnosis of hypogonadotrophic hypogonadism in the transition period was defined in patients who had not yet
reached complete spontaneous puberty in association with low serum sex steroids in the setting of normal or low gonadotropin levels (16,17).

Statistical analysis

Statistical analysis was performed by using the SPSS 13.0 program. Data are expressed as mean ± SD, unless otherwise specified. Nonparametric tests were used because of the non-Gaussian distribution of the data. The differences between the frequencies were evaluated by the Fisher exact test. The differences between the groups were compared by unpaired t test, and not normally distributed two groups were compared by Mann–Whitney U test. P < 0.05 was considered statistically significant.

RESULTS

On the basis of the presence of APA at the first observation in childhood age, the patients were divided in two groups: APA were present with titers ranging from 1:32 - 1:256 in 16 out of 24 patients (66.7%), suggesting the diagnosis of autoimmune hypophysitis (group 1), while they were absent in the remaining 8 patients (group 2). The basal characteristics of APA positive and APA negative patients with GHD at diagnosis are summarized in Table1. In particular, in all sera of patients in group 1, APA immunostained only one type of pituitary cells, identified as somatotrophs by double immunofluorescence. Moreover, the frequency of autoimmune organ-specific antibodies was significantly higher in group 1 than in group 2. Instead, in Table 2 are summarized the values of GH peak, IGF1, height SDS, target height at diagnosis and attained height, GH peak and IGF 1 at re-evaluation in transition period after the stopping of rGH therapy. The relationship between immunological and hormonal characteristics at childhood age and at transition period in patients of group 1 is illustrated in Figure 1. In particular, 4 of them (25%) with presence of APA to
somatotrophs at middle titer (1:32) at childhood age, subsequently, at the re-evaluation in
transition period after stopping rGH therapy, showed disappearance of APA associated with
complete recovery of anterior pituitary function. The other 12 of them (75%), positive for APA
directed to somatotrophs at high titer (> 1:32) at diagnosis, persisted APA positive even if at titer
ranging from 1:16 to 1:32, significantly lower with respect to the starting titers (p < 0.001). As
regards to the type of hormone-secreting cells immunostained by APA in transition period, in eight
out of 12 patients, APA still immunostained selectively somatotrophs with persistence of isolated
GHD. These eight patients showed spontaneous complete puberty and normal MRI characteristics
of hypothalamic pituitary region, except for one of them showing partial empty sella. In the other
four patients (all males) APA did not immunostain somatotrophs, as occurring in childhood, but did
only gonadotrophs (Figure 2). Moreover, at the same time (age range between 20.7 and 21 years) a
recovery of GHD but an incomplete puberty was observed. Therefore, on the basis of testosterone,
LH and FSH levels (testosterone 0.3-1 ng/mL, FSH 0.4-1 IU/L, LH 0.2-0.6 IU/L, respectively),
testis volume ranging between 4 and 6 mL, anamnestic history of delayed puberty at the age of 14-
16 years, without alterations of hypothalamic-pituitary region on MRI nor anosmia, the diagnosis of
isolated autoimmune HH was done. Instead, all eight APA negative patients in group 2 persisted
APA negative in transition period but showing still isolated GHD. Finally, in all normal controls
APA were persistently negative at the start and at the end of the study.

Summarizing, autoimmune GHD, diagnosed and treated since childhood age, recovered in
transition period in four patients in group 1 positive for APA at middle titer at diagnosis, with
disappearance of these antibodies, while persisted in eight out of the remaining 12 patients in this
group, found positive for APA at high titer at diagnosis. Instead, the last four patients in this group
showed, in transition period, a shift of pituitary target of APA from somatotrophs to gonadotrophs
accompanied by recovery of GHD and onset of HH. Moreover, GHD persisted in all negative
patients in group 2 with a significant higher frequency of GHD in these patients with respect to
those in group 1 (p = 0.02).
DISCUSSION

This longitudinal study describes for the first time, in APA positive patients with childhood onset isolated autoimmune GHD treated with rGH replacement therapy, a recovery of pituitary function with disappearance of APA in 25% of them and a shift of APA target (from somatotrophs to gonadotrophs) and of the kind of anterior pituitary dysfunction (from GHD to HH) in other 25% of them in transition age, when GH treatment had been stopped.

The first point emerging from our results was the finding of disappearance of APA with normalization of total anterior pituitary function, when retested at transition period, in four out of 16 patients (25%) with childhood onset autoimmune GHD and presence of APA at middle titer immunostaining selectively the somatothrops at the first observation.

It is already well demonstrated that a possible spontaneous remission during the natural history of autoimmune hypophysitis can be observed and that the recovery of pituitary function occurring after surgical or medical treatment could be related in some cases to spontaneous resolution rather than to the treatment itself (18,19). Thus, a spontaneous remission of pituitary dysfunction in some of our APA positive patients cannot be excluded. However, we can hypothesize that, when APA targeting the somatotrophs are present at middle titer in patients with isolated autoimmune GHD at childhood age, a possible recovery of GH secretion with disappearance of APA can subsequently occur in transition period, if the GH-secreting cells were not irreversibly damaged by the autoimmune process. A similar condition has been reported for some other autoimmune endocrine diseases, in which a remission of the autoimmune process with recovery of the function of the involved hormone-secreting cells was observed, likely because these cells were previously not irreversibly damaged (20,21). Instead, the persisting detection of APA accompanied by impairment of pituitary function in the other 12 patients (75%), in whom APA to somatotrophs had been detected at high titer at diagnosis, seems to indicate that a remission of pituitary autoimmunity in transition period may not occur when the pituitary immune process was very active in childhood
Thus, our results on the hand confirm the usefulness of searching for APA in patients with
GHD in order to disclose those with pituitary autoimmunity, as also demonstrated by other authors
(8,9,18,19,22,23), on the other hand seem to indicate that the titer level of these antibodies at
diagnosis may help to foresee the possible future evolution of the pituitary autoimmune process.
Another interesting point emerging from our results concerns the relationship evidenced at the
observation in transition period between the detection of the type of pituitary hormone-secreting
cells immunostained by APA and the kind of pituitary hormone deficiency in the 12 patients with
persistence of pituitary autoimmunity in this period. Among these patients, eight showed
persistence of APA immunostaining solely somatotrophs, associated with persisting isolated GHD,
while in the other four, pituitary target of APA shifted selectively from somatotrophs to
gonadotrophs with disappearance of GHD and onset of HH.
This is the first evidence in patients with autoimmune GHD, diagnosed in childhood and treated
with rGH replacement therapy until the transition age, of a shift of APA from a pituitary target at
diagnosis (somatotrophs) to another pituitary target (gonadotrophs) at the observation in transition
period, with consequent variation of the selective kind of hypopituitarism, from GHD to HH.
The cause of this surprising behaviour is at present unknown, even because the true pituitary
antigen(s) responsible for the autoimmune response has (have) to be still identified, despite several
factors have been so far suggested as possible autoantigens (22-28).
The results of the present study with the shift of APA target from somatotrophs to gonadotrophs,
accompanied by remission of GHD and onset of HH, seem in part to contradict our previous study
in autoimmune endocrine patients positive for APA but with pituitary function still normal (29), in
which we concluded that, when APA immunostained selectively only one type of pituitary-
secreting cells, were able to foresee the kind of subsequent isolated hypopituitarism. Our present
results seem instead to indicate that a possible shift of the pituitary target of APA with variation of
the kind of hypopituitarism may occur, especially when pituitary autoimmunity started in
childhood. At present we are unable to explain why this may occur. Further studies aimed at
ascertaining the true pituitary antigens evoking the autoimmune response and at investigating
whether any endogenous and/or environmental factor may put on or make silent some of these
antigens from childhood to adult age, could contribute to clarify this aspect.

Instead, the majority of patients in group 1 showed persistently isolated GHD at the observation in
transition period, likely because the presence of APA positive at high titer at diagnosis testified the
occurrence in childhood of a very aggressive immune process, damaging severely GH-secreting
cells, thus not allowing a functional recovery of their function.

In conclusion, our results indicate that patients with autoimmune GHD, diagnosed and treated with
rGH replacement therapy since childhood, may show in transition age, after the stopping of the
treatment, three different conditions, particularly depending on the titer of APA at diagnosis and the
possible variation of the pituitary target of these antibodies from the childhood to the transition age:
i) the majority of them, especially if with APA at high titer at diagnosis, may show a persisting
GHD associated with persisting detection of APA; ii) some of them, with APA at middle titer at
diagnosis, may show a recovery of pituitary function with disappearance of these antibodies, likely
favored by prolonged GH replacement therapy; iii) the remaining patients, even if with autoimmune
process involving in childhood only GH-secreting cells causing isolated GHD, may subsequently
show a shift of APA target from somatotrophs to gonadotrophs, with remission of GHD and onset
of autoimmune HH.

A complete re-evaluation in transition period of pituitary function and of antipituitary antibodies by
double immunofluorescence in patients with GHD, found APA positive in childhood and treated
with replacement therapy, may help to evidence the persistence or the remission of the pituitary
autoimmunity, thus allowing the consequent therapeutic option. Moreover, we suggest that in future
studies this re-evaluation should be made early in occurrence of delayed puberty in some patients,
to allow an early diagnosis and an appropriate therapy in case of autoimmune cross-over from
somatotrophs to gonaadotrophs, thus preventing the progression to future HH.
Declaration of interest: The authors declare that there is no conflict of interest prejudicing the impartiality of the research reported neither any financial or other potential conflict of interest.

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Behavior of antipituitary antibodies (APA) and anterior pituitary function in transition period one year after the stopping of rGH replacement therapy in 24 patients with childhood isolated growth hormone deficiency (GHD).

Detection of pituitary antibodies (APA) by immunofluorescence and characterization of the target of these antibodies by double immunofluorescence in one out of the four patients with GH deficiency at the diagnosis and hypogonadotropic hypogonadism in transition age: cryostat section of young baboon anterior pituitary gland was tested against patient's serum, adding in a primary step FITC-goat antihuman Ig (2a and 2b) and in a second step rabbit antisera anti GH and FSH/LH (2c and 2d), respectively followed by rhodamine-goat sera antirabbit Ig. The close correspondence between the cells colored in green (2a and 2b) and those colored in red (2c and 2d) indicates that the pituitary cells targeted by APA are respectively, GH-secreting cells at diagnosis (2a and 2c) and gonadotropin-secreting cells in transition period (2b and 2d).
Table 1. Basal characteristics of the 24 childhood patients with isolated growth hormone deficiency (GHD)
before the start of recombinant GH therapy

<table>
<thead>
<tr>
<th>Patients</th>
<th>Group 1 (APA positive)</th>
<th>Group 2 (APA negative)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No 16</td>
<td>No 8</td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/6</td>
<td>4/4</td>
<td>ns</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>8.7± 0.54</td>
<td>8.5± 0.69</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>22.2± 1.8</td>
<td>23.1± 1.2</td>
<td>ns</td>
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<tr>
<td>APA titer</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1:32</td>
<td>4</td>
<td>-</td>
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<td>1:64</td>
<td>3</td>
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<td>1:128</td>
<td>7</td>
<td>43.7</td>
<td>-</td>
</tr>
<tr>
<td>1:256</td>
<td>2</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>APA-TPc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH-secreting cells</td>
<td>16</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Other pituitary hormone-secreting cells</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Organ-specific autoimmune diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td>7</td>
<td>43.7</td>
<td>1</td>
</tr>
<tr>
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<tr>
<td>PCAb</td>
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APA: Antipituitary antibodies
PCAAb: parietal-cell antibodies
APA-TPc: Types of pituitary hormone-secreting cells targeted by APA
Table 2. Values of GH peak, IGF1, height SDS, target height at diagnosis and attained height, GH peak, IGF1 at reevaluation in transition period after the stopping of rGH therapy.

<table>
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<th>Patients No</th>
<th>Sex</th>
<th>Age</th>
<th>GH peak (µg/L)</th>
<th>IGF-1 (µg/L)</th>
<th>Height (SDS)</th>
<th>Target Height (cm±8)</th>
<th>Attained height (cm)</th>
<th>GH peak (µg/L)</th>
<th>IGF-1 (µg/L)</th>
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<td>1</td>
<td>M</td>
<td>8</td>
<td>3.07</td>
<td>37</td>
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<td>167</td>
<td>8.1</td>
<td>44</td>
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<td>2</td>
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<td>8.8</td>
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Patient's serum at GHD diagnosis: first step, FITC-goat anti-human Ig

Patient's serum at revaluation: first step, FITC-goat anti-human Ig

Patient's serum at GHD diagnosis: second step, rabbit antiserum anti GH

Patient's serum at revaluation: second step, rabbit antiserum anti FSH/LH