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A Longitudinal Assessment of Hormonal and Physical Alterations during Normal Puberty in Boys II. Estrogen Levels as Determined by an Ultrasensitive Bioassay

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ABSTRACT

A limited number of reports of estrogen levels in prepubertal and early pubertal boys have been published because previous estrogen assays have lacked adequate sensitivity to quantitate circulating estrogen concentrations. Development of a new ultrasensitive assay has permitted measurement of estrogen levels in 23 normally growing boys progressing through puberty. Concentrations were measured at approximately 4-month intervals over a 5- to 8-yr period. The levels increased with maturation in all and correlated directly with chronological age, bone age, weight, height, pubertal stage, and testosterone and insulin-like growth factor-I levels. Of these factors, the level of testosterone had the greatest influence on the estrogen concentration. The time from peak growth velocity also significantly correlated with estrogen level. The estrogen level correlated positively with growth velocity before the time of peak growth velocity and negatively after peak growth velocity. The estrogen levels first increased significantly an average of 3 yr after pubertal onset and reached a peak by 5 yr after pubertal onset. Peak growth velocity was attained an average of 3 yr after pubertal onset. The greatest increase in the rate of rise of the estrogen level was an 11-fold rise during the year in which puberty began. The next most significant increase was a 4.8-fold rise 3 yr after pubertal onset. With respect to pubertal stage, the greatest absolute change occurred from stage 4 to stage 5 and the greatest fold change occurred from stage 1 to stage 2. The estrogen level did not significantly correlate with the 24-h GH level.

In conclusion, circulating estrogen levels are very low in all boys prepubertally and rise steadily during adolescent development. The estrogen level is closely related to testosterone concentration and to the time of peak growth velocity. These findings are consistent with the hypothesis that estrogen at low levels augments skeletal growth and maturation in boys (as well as girls). They are also consistent with the hypothesis that continued exposure to estrogen leads to epiphyseal fusion. Further studies are required to define the separate and combined roles of estrogen, GH, and testosterone, as well as other factors, on growth and sexual development at puberty. (*J Clin Endocrinol Metab* **81:** 3203–3207, 1996)

CIRCULATING estrogen levels have not been previously reported in boys because of the lack of adequate sensitivity of available assays. Previous studies have reported estrogen levels in older prepubertal children of approximately 29.4 pmol/L (8 pg/mL) (1–4). These levels were close to the detection limit of the assay; therefore, they may not be completely reliable. A recent report of estrogen levels in prepubertal girls and boys utilized a new recombinant cell bioassay for estrogen and reported levels of 2.2 pmol/L (0.6 pg/mL) in girls and 0.29 pmol/L (0.08 pg/mL) in boys (5). These data are consistent with the hypothesis that estrogen may exert important biological effects at concentrations well below the minimal detectable concentration in conventional RIAs.

The GH-insulin-like growth factor-I (IGF-I) axis is only one component in the regulation of the pubertal growth spurt. Estrogen may play an important role in the growth spurt in boys as well as girls. The infusion of estradiol at the dose of 4 μg/day triples the ulnar growth rate in boys (6). The plasma estradiol level corresponding to this dose may be approximately 15 pmol/L (4 pg/mL) based on extrapolation from the levels attained at significantly higher dose infusions. Additionally, ethinyl estradiol doses as low as 25–100 ng/kg per day are associated with increased growth velocity in girls with Turner's syndrome (7–9), and doses as low as 100 ng/kg per day are associated with increased GH release in girls with Turner's syndrome (10).

To determine the estrogen levels and the relationships among estrogen level, growth, and GH levels in boys, we used the recently described recombinant cell bioassay to measure estrogen levels in 23 normally growing boys progressing into and through puberty.

Subjects and Methods

Study subjects

Twenty-four normal healthy boys participated in this study. These boys are part of a longitudinal study assessing multiple hormonal and physical alterations during normal puberty. The boys were recruited from the local Charlottesville area and served as paid volunteers. A consent form was signed by a parent or guardian, and assent was received from each boy. The study and consent forms were approved by the Human Investigation Committee at the University of Virginia.

The boys ranged in age from 8.5–12.7 yr at entry. All growth parameters, including weight for height, were between the 5th and 95th percentiles for all boys at entry. None had any chronic illness or was taking medication regularly. Twenty-three of the original 24 boys completed the study. At entry, all were in late pubertal stage 1 or early pubertal stage 2 and reached pubertal stage 5 by the end of the study. The study duration was 5.3–7.4 yr depending on the timing and tempo of pubertal

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development for each boy. All boys progressed through pubertal development in a normal manner during the study.

Study design

The boys were evaluated as inpatients approximately every 4 months. At each evaluation the boys had a physical exam including pubertal staging and height and weight, and a single serum sample was obtained for estrogen, testosterone, and IGF-I at 0600 h. Serial samples were obtained for GH analysis every 20 min for 24 h. Radiographs of the left hand and wrist to assess skeletal maturation were obtained approximately every 8 months. Skeletal maturation was assessed according to the method of Greulich and Pyle (11). Pubertal stage was defined as the mean of pubic hair stage and testicular stage, where pubic hair stage was determined by the method of Tanner and testicular stage was defined as follows: stage 1 included testicular lengths less than or equal to 24 mm, stage 2 included testicular lengths 25–33 mm, stage 3 included testicular lengths 34–37 mm, stage 4 included testicular lengths 38–42 mm, and stage 5 included testicular lengths greater than 42 mm. Growth velocity was calculated over the previous 12 months.

Estrogen assay

The estrogen recombinant cell bioassay was recently described (5). In brief, the assay utilizes a strain of *Saccharomyces cerevisiae*. This yeast was transformed with two plasmids, one containing the human estrogen receptor complementary DNA, and the other containing two copies of an estrogen response element upstream of the yeast iso-1-cytochrome C promoter fused to the structural gene for β-galactosidase. The β-galactosidase activity is assayed to determine estrogen concentration. The sensitivity of the bioassay was 0.07 pmol/L (0.02 pg/mL). The increased sensitivity of the assay comes at least in part from the overexpression of estrogen receptor in the system, the high amplitude response produced by the tandem arrangement of the two estrogen response elements, and the low background in the absence of estrogen. The assay was highly specific for estradiol. The intraassay coefficient of variation (CV) at 7.4 pmol/L (2 pg/mL) was 15%. The interassay CV at 7.4 pmol/L was 13%.

GH assay

Serum GH concentrations were determined using Nichols Institute (San Juan Capistrano, CA) immunoradiometric assay kits with horse serum matrix. All samples from any one study period were analyzed in the same assay run, with samples from as many study days as possible for any one child assayed on the same day. Intra- and interassay CVs were less than 8% and 10%, respectively. The lower limit of assay sensitivity was determined to be 0.5 μg/L, and all samples with GH concentrations less than this were set equal to 0.5 μg/L for analysis.

Testosterone assay

Serum testosterone levels were determined using a commercial solid-phase RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The minimal detectable dose was 0.70 nmol/L

IGF-I assay

Plasma IGF-I levels were assayed following acid-ethanol extraction in kits from the Nichols Institute using a double antibody RIA. The intra- and interassay CVs were less than 5.2% and 11.2%, respectively, with a lower limit of sensitivity of 34 ng/mL.

Statistical analysis

Estrogen, testosterone, and mean 24-hour GH levels were first averaged for each pubertal stage for each boy, and then averages were calculated for each stage for all boys to eliminate the influence of differing numbers of data points for each boy in each pubertal stage. This procedure was done to account for the differing tempo of puberty. Estrogen, testosterone, and mean 24-hour GH levels were also analyzed relative to pubertal onset defined as the age at the visit when any sign of puberty was first noted, and also relative to the time of peak growth velocity for each boy. Means across all groups were compared by ANOVA. Means between each pair of groups were compared by the Student's t test. Correlations were assessed by linear regression and by multivariate analysis using multiple ANOVA. Statistical significance was accepted at $P < 0.05$.

Results

Estrogen

The estrogen level increased with advancing pubertal stage in all boys (Table 1 and Fig. 1). The increase across all stages was significant, but the only significant increase between individual stages was between stages 4 and 5. The greatest absolute increase in estrogen level relative to pubertal stage was between stages 4 and 5. The greatest rate of increase was an 11-fold rise between stages 1 and 2.

The estrogen level also increased significantly with increasing chronological age (data not shown), increasing bone age (Fig. 2), increasing time relative to pubertal onset (Fig. 3), and increasing time relative to peak growth velocity (Fig. 3). Changes from year to year relative to chronological age were not statistically significant. Changes year to year relative to bone age only became significant from bone age 13–14 yr.

The first significant change in the estrogen level occurred during the year immediately preceding peak growth velocity (Fig. 3A), which was approximately 3 yr after pubertal onset (Fig. 3B). The estrogen level also increased significantly during the year following that of peak growth velocity. The greatest absolute changes in the estrogen level also occurred during the year following attainment of peak growth velocity, and 5 yr after pubertal onset. The greatest fold changes in estrogen occurred during the year immediately preceding peak growth velocity, a 4-fold rise, and during the year immediately preceding pubertal onset, an 11-fold rise.

The estrogen level correlated significantly with bone age, weight, height, testosterone, IGF-I, time from pubertal onset, and time from peak growth velocity (Figs. 4 and 5). The strongest univariate correlation was with testosterone level. Testosterone also had the most influence on the estrogen level as determined by multivariate analysis including pubertal stage, peak growth velocity, time of pubertal onset, body mass index (defined as weight in kilograms divided by

TABLE 1. Hormone concentrations by pubertal stage in boys

Stage	E_2 (pmol/L)[a]	T (nmol/L)	GH (μg/L)	GV (cm/yr)
1	0.14 ± 0.03	0.84 ± 0.01	4.23 ± 0.30	5.24 ± 0.50
2	2.28 ± 0.73	1.80 ± 0.21	3.40 ± 0.20	5.61 ± 0.13
3	5.21 ± 1.73	9.51 ± 1.03	4.72 ± 0.50	6.78 ± 0.24
4	10.7 ± 2.13	13.9 ± 0.66	5.47 ± 0.40	8.16 ± 0.24
5	42.5 ± 3.27	20.0 ± 0.36	3.81 ± 0.20	5.03 ± 0.24

[a] Mean ± SE; E_2, estrogen, T, testosterone, GV, growth velocity.

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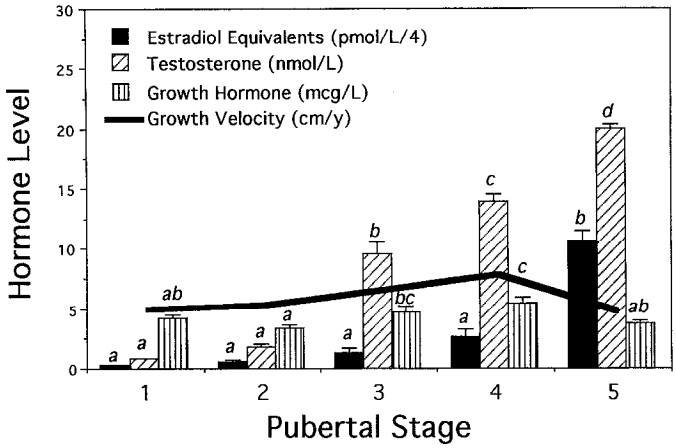

FIG. 1. Mean estrogen level (*solid bars*), mean testosterone level (*hatched bars*), mean 24-h GH level (*striped bars*), and growth velocity (*solid line*) in boys at each stage of puberty. Estrogen levels are divided by 4 for purposes of presentation with other hormones. Growth velocity (cm/yr) is indicated on the same y-axis as hormone levels. Bars representing the same hormone but labeled with *different letters* are significantly different at the $P < 0.05$ level. *Similar letters* indicate no significant difference.

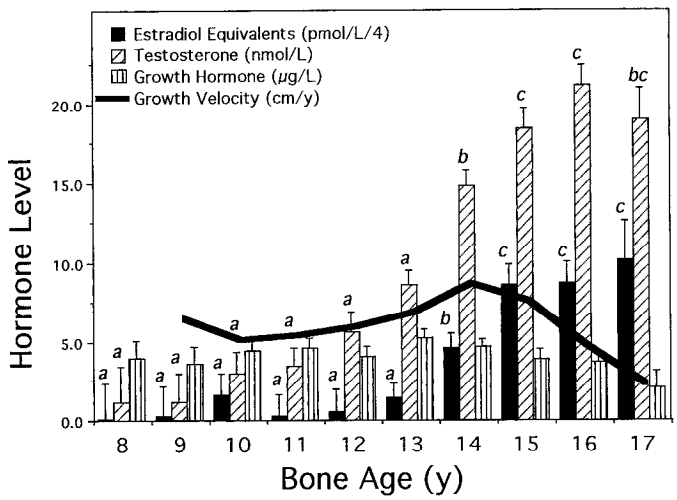

FIG. 2. Mean estrogen level (*solid bars*), mean testosterone level (*hatched bars*), mean 24-h GH level (*striped bars*), and growth velocity (*solid line*) in boys relative to bone age. Estrogen levels are divided by 4 for purposes of presentation with other hormones. Growth velocity is indicated on the same y-axis as hormone levels. Bars representing the same hormone but labeled with *different letters* are significantly different at the $P < 0.05$ level. *Similar letters* indicate no significant difference.

the square of the height in meters), GH, IGF-I, and chronological age (data not shown).

The estrogen level and mean 24-h GH level did not significantly correlate.

GH

Mean 24-h GH level increased slightly to a peak at stage 4 of puberty coincident with the peak growth velocity (Table 1 and Fig. 1). Mean 24-hour GH level first increased significantly between −2 and −1 yr before peak growth velocity (Fig. 3A) and 2 yr after pubertal onset (Fig. 3B). Mean GH level also increased significantly between −1 and 0 yr before

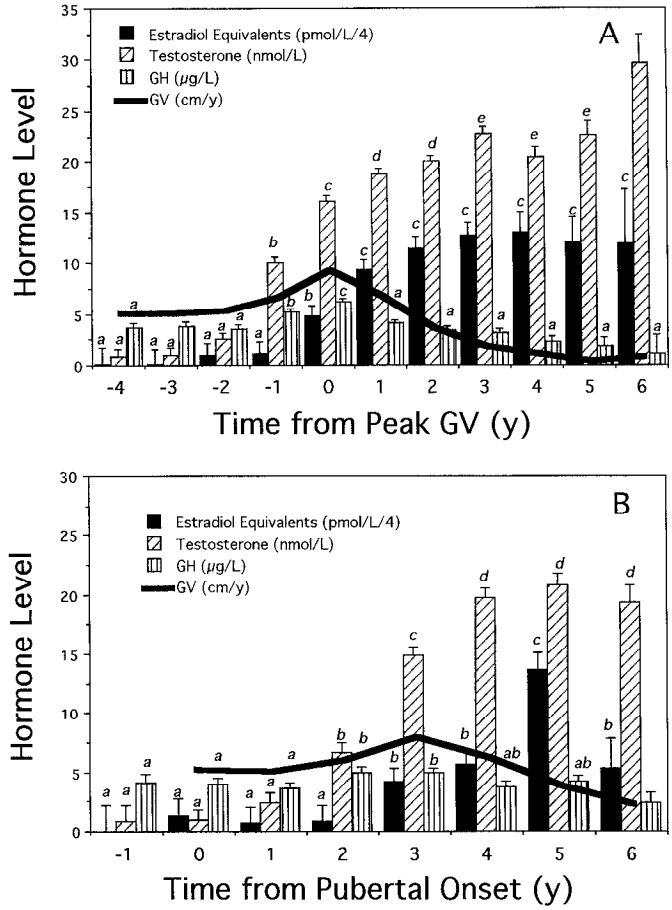

FIG. 3. Mean estrogen level (*solid bars*), mean testosterone level (*hatched bars*), mean 24-h GH level (*striped bars*), and growth velocity (*solid line*) in boys relative to year from peak growth velocity (A), and relative to year from pubertal onset (B). Estrogen levels are divided by 4 for purposes of presentation with other hormones. Growth velocity is indicated on the same y-axis as hormone levels. Bars representing the same hormone but labeled with *different letters* are significantly different at the $P < 0.05$ level. *Similar letters* indicate no significant difference.

peak growth velocity but decreased significantly between 0 and 1 yr after peak growth velocity. It continued to decrease each subsequent year, although this downward trend did not reach statistical significance. Peak GH level occurred during the year of peak growth velocity and 2 and 3 yr after pubertal onset.

Testosterone

The testosterone level first increased significantly between −2 and −1 yr before peak growth velocity (Fig. 3A) and 2 yr after pubertal onset (Fig. 3B). The testosterone level also increased significantly between −1 and 0 yr before peak growth velocity, between 0 and 1 yr after peak growth velocity, and 3 and 4 yr after pubertal onset.

Multivariate analysis

Of all the parameters considered, the testosterone concentration and the mean GH level had the greatest influence on peak growth velocity and on pubertal onset using multivar-

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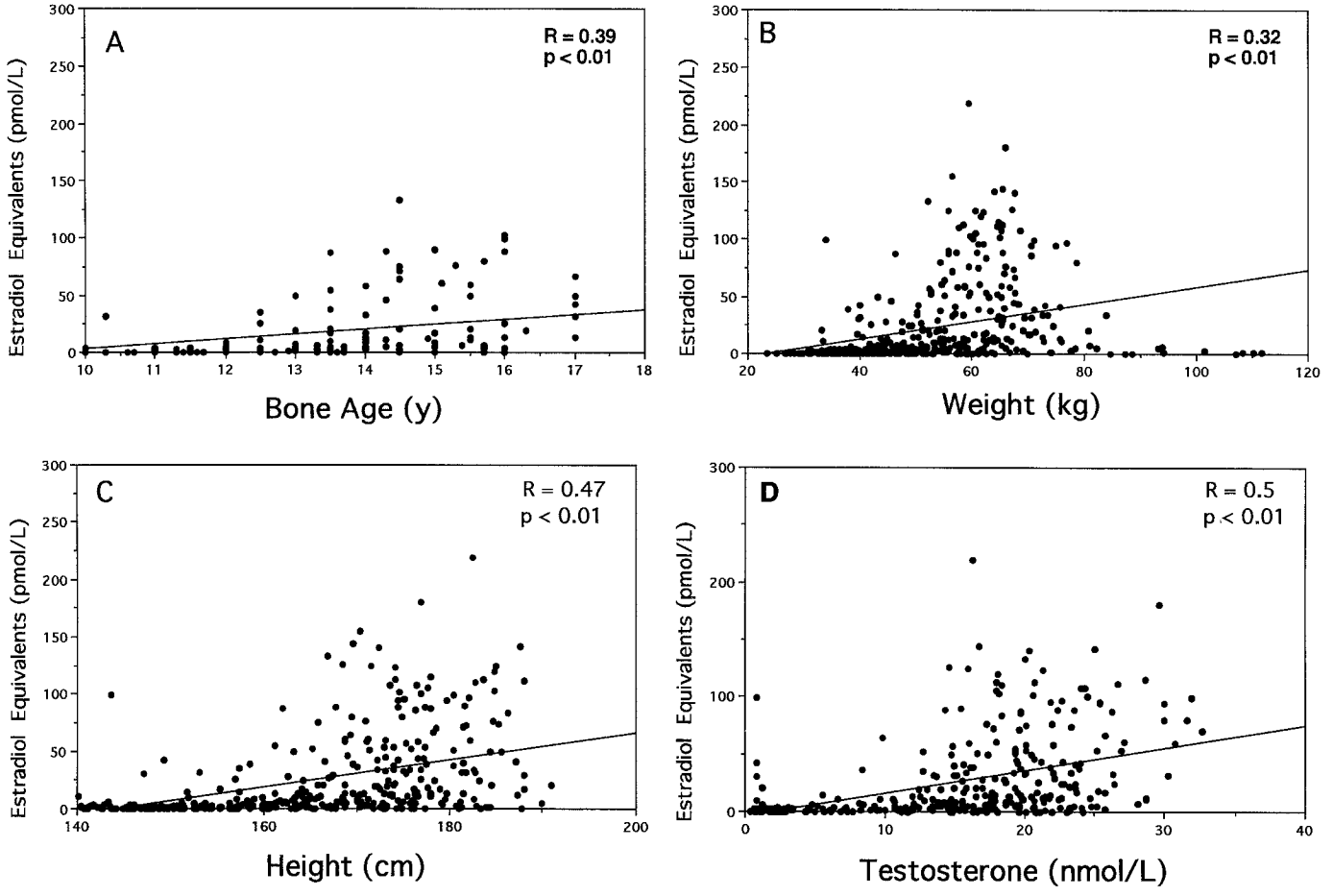

FIG. 4. Simple linear correlations between estrogen level and bone age (A), weight (B), height (C), and testosterone level (D) in boys as they develop through puberty. Each subject (n = 23) is represented at each visit (12–18 visits).

iate analysis; however, there was an additional component influencing the timing of peak growth velocity accounted for only by the estrogen concentration (Table 2). The time relative to peak growth velocity was a better predictor of the estrogen level than was the time relative to pubertal onset using multivariate analysis (Table 2).

TABLE 2. Multivariate analysis[a]

Source of effect	F ratio	P
Hormone influence on peak growth velocity		
Testosterone	571	<0.001
GH	28.1	<0.001
Estrogen	4.41	0.03
Parameter influence on estrogen concentration		
Time from pubertal onset	0.01	0.94
Time from peak growth velocity	10.2	0.002

[a] Where F ratio is the statistic testing the effect is 0, and P is the probability that the effect is random error.

Discussion

Most previous studies of circulating estrogen levels in boys have been limited by the sensitivity of available assays (1–4). We describe estrogen levels as normally growing boys progress into and through puberty, using a recently described recombinant cell bioassay. The first significant rise in the estrogen level occurred simultaneously with the peak growth velocity, an average of 3 yr after pubertal onset. This relationship between peak growth velocity and the rise in estrogen level further supports the hypothesis that estrogen at low levels may be a mediator of skeletal growth and maturation in boys as well as girls. The estrogen levels continued to increase and remained at the relatively higher levels toward the end of puberty, although growth velocity decreased as epiphyseal closure became imminent. This pattern is consistent with the hypothesis of a biphasic effect of estrogen on growth velocity, in which low levels accelerate growth in concert with increasing testosterone and GH levels, and high levels accelerate epiphyseal fusion, although these hypotheses were not directly tested in this study. It is also consistent with the hypothesis that sustained levels of estrogen over time lead to epiphyseal fusion, and with the recent description of a man with estrogen resistance because of a mutant estrogen receptor, who had tall stature and incompletely fused epiphyses late in his third decade (12).

Testosterone levels rose during puberty in all boys. The first significant rise occurred 1 yr before peak growth velocity. Thus, the initial significant changes in estrogen level lagged the changes in testosterone by 1 yr.

The highest mean GH levels occurred simultaneously with

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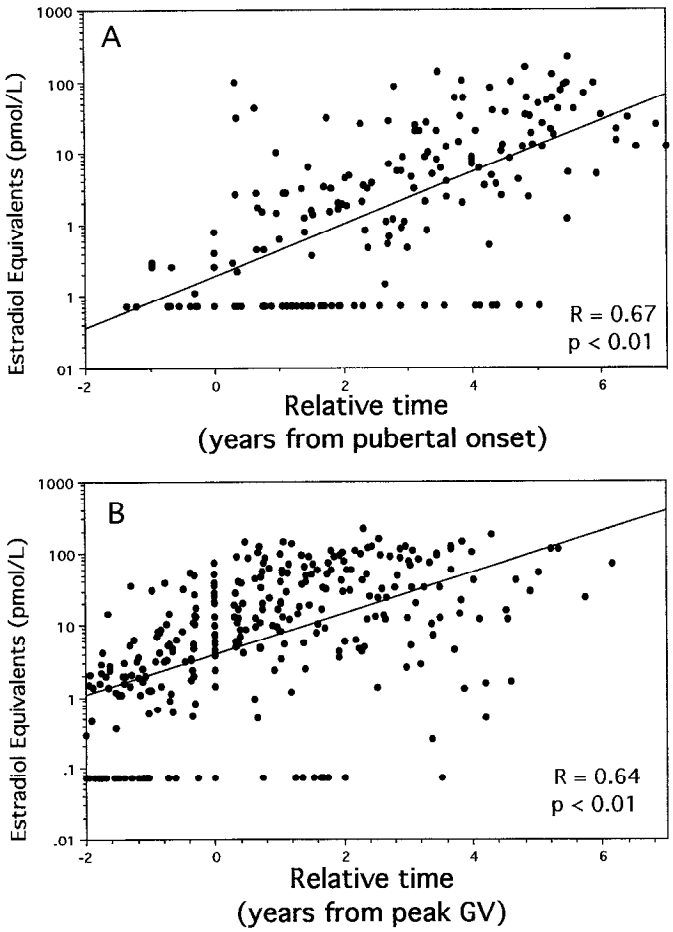

FIG. 5. Simple linear correlations between estrogen level and year from pubertal onset (A) and year from peak growth velocity (B) in boys as they develop through puberty.

the peak growth velocity as previously described (13), although this is the first longitudinal confirmation of the previous cross-sectional observations. This suggests a relationship between GH and sex steroid secretion, and also suggests that GH is one of the major mediators of the pubertal growth spurt. Similar to the testosterone level, the first significant rise in mean GH level occurred in the year prior to peak growth velocity.

Direct effects of androgens and estrogen on growth as well as indirect effects of estrogen on growth mediated by augmented secretion of GH have been described (14). Androgens do not appear to play as much a role in bone age maturation and the pubertal growth spurt as do estrogens as described in boys treated with oxandrolone (15), in the description of a genetic female with pseudohermaphroditism caused by an aromatase-gene defect in whom bone age was delayed and there was no pubertal growth spurt (16), and in boys with familial male precocious puberty, who may require treatment with aromatase inhibitors as well as antiandrogens to slow skeletal growth and bone maturation (17). Estrogen is also involved in the cessation of growth, or epiphyseal fusion, in boys and girls. The estrogen levels described in this study are consistent with direct and indirect roles for estrogen, as well as a role in epiphyseal closure. Patients with complete androgen insensitivity have a pubertal growth spurt that is normal for a genetic female (18).

We conclude that estrogen levels are very low in all boys prepubertally, begin to increase even before the external signs of puberty are obvious, and then rise steadily during the remainder of adolescent development. The circulating estrogen level is closely related to testosterone concentration and to the time of peak growth velocity. These findings are consistent with the hypothesis that estrogen at low levels influences skeletal growth and maturation in boys (as well as girls). These findings are also consistent with the hypothesis that continued exposure to estrogen accelerates epiphyseal fusion. Further studies are required to define the hormonal mechanisms of estrogen action at puberty.

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