0021-972X/96/$03.00/0
Journal of Clinical Endocrinology and Metabolism
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Vol. 81, No. 2
Printed in U.S.A.

Mullerian Inhibiting Substance in Humans: Normal Levels from Infancy to Adulthood*

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ABSTRACT

Mullerian inhibiting substance (MIS) is a gonadal hormone synthesized by Sertoli cells of the testis and granulosa cells of the ovary. To facilitate the use of MIS for the evaluation of intersex disorders and as a tumor marker in women with MIS-expressing ovarian tumors, we measured MIS in 600 serum samples from males and females. These data show that mean MIS values for males rise rapidly during the first year of life and are highest during late infancy, then gradually decline until puberty. In contrast, MIS values in females are lowest at birth and exhibit a minimal increase throughout the prepubertal years. Whereas MIS is uniformly measurable in all prepubertal boys studied, it is undetectable in most prepubertal female subjects. These data reveal an easily discernible sexually dimorphic pattern of expression and confirm that MIS can be used as a testis-specific marker during infancy and early childhood. MIS values that are above the upper limits for females are discriminatory for the presence of testicular tissue or ovarian tumor, and those below the lower limits for males are consistent with dysgenetic or absent testes or the presence of ovarian tissue. These data will enable normal and abnormal levels of MIS to be differentiated with higher precision and will facilitate the use of MIS in the management of gonadal disorders. (*J Clin Endocrinol Metab* **81:** 571–576, 1996)

A MAJOR FUNCTION of Mullerian inhibiting substance (MIS; also known as anti-Mullerian hormone) is to cause involution of the Mullerian ducts during male embryogenesis (1). MIS is synthesized by Sertoli cells of the testes prenatally and throughout life and is also produced by granulosa cells of the ovary after birth (reviewed in Refs. 2 and 3). In males, the ontogeny of MIS expression is similar across species, in that MIS is expressed at higher levels in prepubertal than in pubertal or adult testes in all species examined (4–6). In the female rodent, levels of MIS protein and messenger ribonucleic acid, which are negligible in the prepubertal ovary, increase modestly at puberty (6–8). In humans, previous data indicated that circulating MIS is variably measurable in adult women, but undetectable in prepubertal girls by enzyme-linked immunosorbent assay (ELISA) (9). These studies confirmed that MIS expression is clearly sexually dimorphic during sexual immaturity. Consequently, the determination of MIS during infancy and the early prepubertal period as a marker of Sertoli cell function in the male would be useful for delineating testicular from ovarian tissue (10, 11). We had also previously described the clinical utility of MIS determination as a tumor marker for selected granulosa and sex cord tumors that secrete supraphysiological levels of MIS (12).

Several assays are currently available for the measurement of MIS in serum or plasma (9, 13, 14). These assays are all highly sensitive and specific for primate MIS, but differ in the particular preparation of recombinant human MIS used for the standards and the unique antibodies used in the assays. Therefore, measured MIS values from the same samples vary in the 3 established MIS assays (Hudson, J. M., MacLaughlin, D. T., and Donahoe, P. K.), and normative data for circulating MIS concentrations need to be generated for each specific assay. To facilitate the use of serum MIS determination for the clinical diagnosis and management of intersex disorders and the monitoring of tumor recurrence in women with MIS-expressing ovarian tumors, we expanded the normative data for MIS across all age ranges in males and females. Because of the wide variations in MIS values in the limited number of normal subjects studied previously (9, 13, 14), we collected 600 serum specimens across a wide range of ages (from newborn to adult) to generate normative data for MIS. We now report normal levels and 95% normal limits for serum MIS concentrations as

Received May 9, 1995. Revision received August 16, 1995. Accepted September 8, 1995.

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* Presented in part at the Meeting of the Fourth Joint Lawson Wilkins Pediatric Endocrine Society/European Society for Pediatric Endocrinology, San Francisco, CA, 1993, and at the Japanese Pediatric Endocrine Meeting in Tokyo, Japan, 1994. This work was supported in part by grants from the Genentech Foundation for Growth and Development and the NIDDK (DK-02129; to M.M.L., General Clinical Research Center Grant MO1-RR-01066, FDA Grant FD-R-000669 (to D.T.M.), NCI Grant CA-17393 (to D.T.M. and P.K.D.), and NICHD Grant P30-MD-28138 (to M.M.L., P.K.D., and D.T.M.).

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a function of chronological age using 375 data points from 288 male subjects and 225 data points from 188 female subjects.

Subjects and Methods

Study population

The serum samples were obtained primarily from four centers: Massachusetts General Hospital (Boston, MA), Tokyo Metropolitan Kiyose Children's Hospital (Tokyo, Japan), Children's Memorial Hospital (Chicago, IL), and New York Medical College (Valhalla, NY). The subjects were predominantly healthy individuals with no endocrine or systemic illnesses. A minority of the control subjects were children with genetic short stature or adequately controlled hypothyroidism followed in one of the endocrine clinics. The ages ranged from 1 day to 50 yr. After sample collection, serum was separated and stored at −20 C until assayed in the MIS ELISA, as described below. The serum aliquots were assigned a sequential number to protect patient anonymity. The control data were comprised of both cross-sectional and longitudinal sampling from the normal subjects. MIS levels were determined in 375 samples (88 serial specimens) from 288 control male subjects and 225 samples (46 serial specimens) from 179 control female subjects. All samples in this study were collected under the guidelines of the respective institutional human studies internal review board.

To evaluate the effects of long term storage and repeated freeze-thaws on the measurement of MIS, 35 samples were reassayed after varying periods of long term storage at −20 C and selected freeze-thaws. To verify the day to day stability of circulating serum MIS concentrations, duplicate samples were obtained from five prepubertal boys between the ages of 5–10 yr at intervals of several days to weeks.

MIS ELISA

The ELISA used to measure human MIS is similar to that described previously (9). The protein A-purified 6E11 mouse monoclonal antibody to recombinant human MIS (rhMIS) was diluted to a concentration of 10 μg/mL in 0.05 mol/L sodium carbonate-bicarbonate buffer (pH 9.6) and plated at 50 μL/well on Dynatech Immulon II 96-well plates. The plates were incubated for 2 h at room temperature, then blocked for 2 h with 5% MIS-free female FCS (Biologos, Naperville, IL) in phosphate-buffered saline (PBS). Next, either 50 μL rhMIS at concentrations of 0.1–20 ng/mL for the standard curve or 50 μL serially diluted serum samples were added for an overnight incubation at 4 C. The following morning, protein A-purified MGH-4, a rabbit polyclonal antibody that recognizes rhMIS, was added at a 1:10,000 dilution in 5% female FCS in PBS and incubated for 1 h at room temperature. The plates were then incubated with mouse antirabbit horseradish peroxidase (Accurate Chemical and Scientific Corp., Westbury, NY) at a 1:3,000 dilution in 5% female FCS in PBS at 4 C for 1 h. Fifty microliters per well of 42 mmol/L tetramethyl benzidine in dimethylsulfoxide, diluted 1:100 in 0.1 mol/L citrate buffer (pH 4.9) with 50 μmol/L hydrogen peroxide, were added to visualize the complexes. After 12.5 min, the reaction was stopped by adding 50 μL 2 N sulfuric acid. All of the PBS washes between incubations were performed with a Biotek autowasher (Winooski, VT) programmed to complete six washes of 150 μL each. A Biotek microplate reader was used to measure the optical density at 450 nm, from which DeltaSoft II (BioMetallics, Princeton, NJ) software fits a four-parameter standard curve and interpolates the concentration of the unknown serum samples.

Serum samples were analyzed in duplicate at six serial dilutions; the results reported were the mean of three dilutions falling within the linear portion of the standard curve, constructed using four parameter logistical curve fitting. The sensitivity of this assay is 0.5 ng/mL; the intra- and interassay coefficients of variation are 9% and 15%, respectively. The MIS ELISA does not recognize LH, FSH, activin, inhibin, or transforming growth factor-β and does not cross-react with bovine or rodent MIS (9), but does recognize nonhuman primate MIS (5).

Statistical analysis

The data were divided into two groups; one group consisted of values taken at ages less than 1 yr, and the other group consisted of values taken at ages greater than 1 yr. MIS values and ages were log transformed using $\log_2$. The mean value of log(MIS) was assumed to be a quadratic function of $\log_2$, *i.e.* $\log(\text{MIS}) = a \log(\text{age}) + b + c \log(\text{age})^2$, where a is the change in MIS for every doubling of age, b is the value at birth, and c is the quadratic correction factor. Because several patients had multiple measurements, the model for the variation in MIS had to account for the correlation between a given patient's values. The model assumed that the SD within a patient was of the form $\sigma_{\text{error}} \times (\text{age})^f$, where the factor $(\text{age})^f$ was introduced to allow for the fact that the dispersion increased with age. The model assumed an additional between-patient SD of log (MIS), which is σ_{subject}.

The estimation was performed in two steps. First, we used maximum likelihood estimation to find f, ignoring the fact that there were multiple measurements taken per subject. Then, we used the estimated values of f in a mixed model ANOVA to estimate the other parameters. Due to MIS values equal to zero, one was added to each female value before performing log transformations. The formula for predicting MIS is: $\log(\text{MIS}) = a \log(\text{age}) + b + c \log(\text{age})^2$.

The formula for upper and lower normal limits for log (MIS) follow:

$$U = g(\text{age}) + 1.98\sqrt{\sigma^2_{\text{subject}} + (\sigma_{\text{error}} \times (age)^f)^2} \quad (1)$$

$$L = g(\text{age}) - 1.98\sqrt{\sigma^2_{\text{subject}} + (\sigma_{\text{error}} \times (age)^f)^2} \quad (2)$$

where g (age) is the predicted value, given the above formula for MIS prediction. Normal limits for specifized age ranges are given. For each interval, we calculated the predicted upper and lower 95% confidence limits for the ages in our data that fell into this interval (see Tables 1, 3, and 4). The means and medians for these age ranges are also listed in the tables. Thus, the values reflect the distribution of ages in the data. The tables contain MIS values transformed back to their original scale of nanograms per mL.

Results

Assay variables

To assess the effects of long term storage and freeze-thawing of the samples, 35 samples were frozen for periods varying from 1 month to 4 yr, then reassayed in the MIS ELISA. In addition, several of the samples were subjected to repeat freeze-thaws. These procedures confirmed that MIS is relatively stable when stored at −20 C for up to 2 yr and tolerated up to three freeze-thaws without a significant change in measured MIS concentrations (data not shown). In serum stored for less than 2 yr, the fluctuation in MIS values was within the 15% interassay variability of the assay. However, MIS values increased by up to 2- to 3-fold after 2 yr of storage at −20 C and decreased by at least 50% after three freeze-thaws.

Patient variables

Analysis of variance of serial samples in five patients showed that the intrapatient variability expressed as a proportion of the mean was approximately 15% in samples obtained several days apart and analyzed in the same assay. Thus, with a 9% intraassay variability of the assay (9), the intrapatient variability was only 10%.

MIS values for age less than or equal to 1 year

Table 1 shows the means, medians, and normal limits for males and females under 1 yr of age. Parameter values are listed in Table 2. Figure 1 is a scatterplot of the MIS values in males and females less than 1 yr of age on a $\log_{10}$ scale with 95% normal limits plotted for comparison. At birth, MIS values were low, but rose rapidly during the first week of life to mean levels 4-fold higher by 3–4 days of age. Mean values for males continued to rise rapidly during the first half year

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TABLE 1. MIS values (nanograms per mL) for males and females less than 1 yr of age

Age (days)	n	Mean	Median	Lower limit	Upper limit
Males					
1–3	6	6.8	6.0	3.1	48.1
3	33	22.4	17.3	3.7	58.0
4–30	29	26.0	24.3	7.8	121.5
31–60	13	55.0	48.0	11.2	174.2
61–120	21	60.9	47.0	14.1	218.5
121–180	27	115.5	96.3	15.7	243.8
181–240	17	81.0	67.0	18.7	289.8
241–300	15	82.7	55.0	19.9	307.8
300–365	13	60.8	50.0	21.7	336.3
Females					
1–365	53	0.66	0.48		1.9

TABLE 2. Parameter estimates

Parameter	Age <1 yr		Age >1 yr	
	Male	Female	Male	Female
Change in MIS for every doubling of age (a)	0.367	−0.013	0.238	1.993
Value at birth (b)	5.101	0.558	6.004	−4.848
Quadratic correction factor (c)	0.000	0.000	−0.258	−0.151
$\sigma_{subject}$	0.735	0.394	1.118	0.555
σ_{error}	0.675	0.324	0.616	0.435
f	0.000	0.130	0.101	0.058

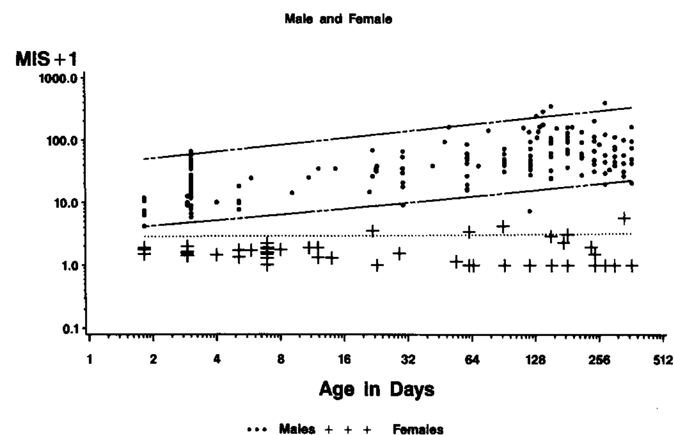

FIG. 1. Serum MIS levels (nanograms per mL) in 174 male and 53 female infants 1 yr of age or younger are plotted on a $\log_{10}$ scale for MIS *vs.* postnatal age in days. No overlap of normal levels was found in this age range. Also shown are the upper and lower 95% normal limits for the males (*dashed lines*) and the upper 95% normal limits for the females (*dotted line*), derived from the mathematical model described in *Materials and Methods*. Note that all 174 male infants in this age range had easily measurable serum MIS, and the MIS levels in the female infants were much lower. Approximately half of the 53 female cases (n = 26) had serum MIS levels of 0.5 ng/mL (lower limit of detection of the assay) or less upon initial assay and before the mathematical transformations for statistical analyses.

of life and started to decline during late infancy. Values in females were low during the first year of life, and approximately half of the 53 female cases (n = 26) had serum MIS levels less than or equal to 0.5 ng/mL (lower limit of detection of the assay).

For males, the mean of the log-transformed MIS was a linear function of log (*age*), that is parameter *c* is equal to 0. Further, $\sigma_{error} \times (\text{age})^f$ was constant. Therefore, normal limits could be expressed simply. The lower limit for MIS values at 2 months was 11.2 ng/mL. For every doubling of age, the lower limit of MIS increased by one third. For every halving of age, the lower limit decreased by one third.

For females, the mean of the log-transformed MIS was also a linear function of log (*age*). The upper limit for MIS values at 2 months was approximately 1.6. For every doubling or halving of age, the upper limit increased (or decreased) slightly (1/100th).

MIS values for age greater than 1 year

Tables 3 and 4 contain the means, medians, and normal limits for males and females greater than 1 yr of age. Parameter values are listed in Table 2. Figure 2 is a scatterplot of both male and female data with 95% normal limits on a $\log_{10}$ scale. Mean values in male subjects were highest in infancy, then gradually declined until puberty when they overlapped extensively with values in pubertal female subjects. The range of values in males was greatest at 1–4 yr of age. In contrast, MIS values in females were lowest and typically undetectable during infancy, with a minimal rise throughout childhood and at puberty. Figures 1 and 2 show the lack of overlap in the 95% normal limits for MIS in males and females before the age of 3 yr. Starting at ages 3–4 yr, the lower limits for boys overlapped with the upper limits for girls, although the mean and median values remained at least 10-fold higher in boys. By puberty, there was extensive overlap of normal MIS levels.

For males and females, the mean of the log-transformed MIS was a quadratic function of log(*age*).

Longitudinal data on male infants

Figure 3 is a plot of the individual MIS values with increasing age in five representative male infants during the first few years of life. MIS values consistently increased after birth to peak between 6–12 months of age. Although absolute MIS values varied widely between individuals, all five subjects had a similar longitudinal pattern of expression. Not all individuals studied had this same pattern of expression; some boys did not have such a marked increment in MIS during late infancy.

Discussion

In humans, as in other species (4–7), the expression of MIS is age and gender specific from infancy to adulthood. We have determined upper and lower 95% normal limits, mean,

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TABLE 3. MIS values (nanograms per mL) for males greater than 1 yr of age

Age (yr)	n	Mean	Median	Lower limit	Upper limit
1–2	41	91.9	54.0	9.9	444.1
2–4	20	71.1	56.3	7.4	373.1
4–6	13	45.7	33.0	4.9	264.5
6–8	14	55.7	33.5	3.2	182.4
8–10	15	37.2	32.3	2.3	139.1
10–12	17	41.3	26.0	1.7	104.5
12–14	14	6.9	5.1	1.3	83.9
14–16	18	5.9	4.7	1.1	69.4
16–18	6	3.7	2.5	0.7	47.3
18–32	31	4.4	2.9	0.3	25.0
>32	12	4.0	1.6	0.1	9.6

TABLE 4. MIS values (nanograms per mL) for females greater than 1 yr of age

Age (yr)	n	Mean	Median	Upper limit
1–2	19	0.9	0.6	3.9
2–4	13	1.4	1.2	6.8
4–6	13	1.4	1.2	8.1
6–8	17	3.0	2.9	8.8
8–10	14	2.3	2.1	8.9
10–12	16	2.7	2.5	8.8
12–14	12	3.1	2.6	8.6
14–16	31	4.7	2.4	8.3
16–18	8	2.1	2.0	8.1
18–32	23	1.7	1.5	7.0
>32	6	0.9	1.2	5.4

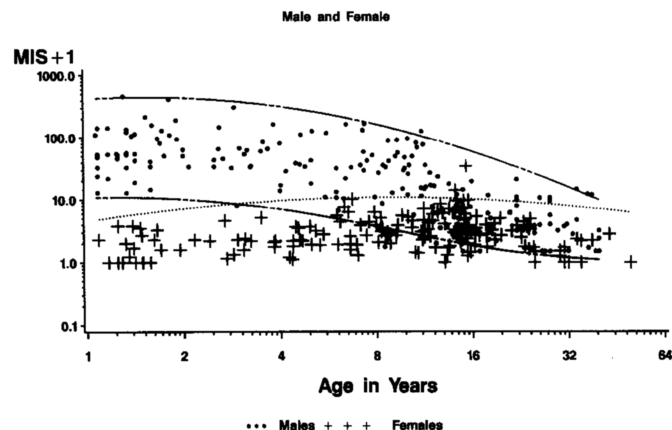

FIG. 2. Serum MIS levels of male (n = 201) and female (n = 172) subjects greater than 1 yr of age are plotted on the same graph using a $\log_{10}$ scale for MIS. The lower 95% normal limit for males (*dashed line*) falls to undetectable levels at approximately 18 yr. Thus, with this mathematical model, 2.5% of normal males 18 yr or older will have undetectable serum MIS. The upper 95% normal limits of the determinations for females appear as a *dotted line*. Unlike the younger age group (see Fig. 1), only 31 of the 172 cases (18%) had MIS levels at or below the limit of MIS detection in the assay. Although values in males and females start to overlap by 3 or 4 yr of age, mean and median values at all prepubertal ages remain significantly different.

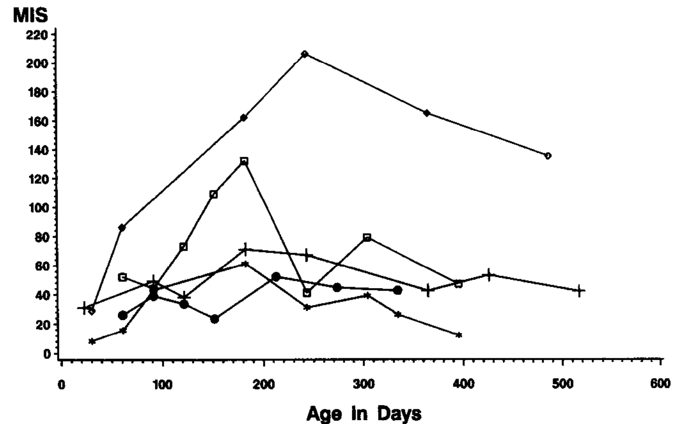

FIG. 3. Longitudinal serum MIS data for five males collected during the first 2 yr after birth show the same trends depicted in the cross-sectional data for males, that is a rise and subsequent fall in serum MIS between 6 (180 days) and 12 months (365 days) of age.

and median serum MIS concentrations in a large number of male and female subjects at specified ages using the MIS assay established in the Pediatric Surgical Research Laboratory at the Massachusetts General Hospital. These data depict the characteristic pattern of MIS secretion from infancy to adulthood and verify that in normal male subjects, peripheral MIS concentrations vary extensively between individuals. In contrast, MIS values in an individual subject exhibit age-related changes and remain within consistent ranges longitudinally. Whereas MIS is uniformly detectable in the circulation of all prepubertal boys studied, it is below the limits of assay sensitivity (<0.5 ng/mL) in many female subjects at all ages. Circulating MIS concentrations in females are tightly clustered at the lower detectable limits of the assay, with no values above 10 ng/mL in any of the 225 female specimens. In the population of female subjects we studied, the mean serum MIS values rise during late puberty, then decline as a function of age. We have initiated additional studies to clarify the changes in MIS with aging, particularly during the perimenopausal period as ovarian function declines. The presence of low levels of circulating MIS in females is consistent with immunohistochemical and molecular studies demonstrating ovarian expression of MIS in several species (7, 8, 15, 16). Thus far, the two other published assays for MIS (13, 14), although having the same specificity and similar lower limits of sensitivity, have not been able to measure the low levels found in female subjects.

These data reveal an easily discernible sexually dimorphic pattern of expression and confirm that MIS can be used as a testis-specific marker during infancy and early childhood. Circulating MIS levels clearly distinguish males from females before the age of 3 yr, with no overlap in normal values. Moreover, mean and median MIS values continue to be at least 10- to 20-fold higher in males than females until

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the age of 12 yr. Therefore, these data indicate that MIS values that fall above the upper limit for females are discriminatory for testicular tissue (or the rare exception of a MIS-secreting ovarian tumor) (12). Likewise, a measurable value below the lower limits for males is indicative of either ovarian or dysgenetic testicular tissue (10), and an unmeasurable value is consistent with absent gonads, ovaries, or dysgenetic/streak gonads (10). By the age of 3–6 yr, however, values within the lower ranges for boys start to overlap with the higher values seen in girls, and values that fall within this area of overlap are nondiagnostic.

The MIS assay established by the Pediatric Surgical Research Laboratory at Massachusetts General Hospital is a sandwich technique employing a monoclonal and a polyclonal antibody to rhMIS and is highly specific for full-length unprocessed MIS. Full-length MIS is proteolytically processed to an active carboxy-terminal fragment and an inactive amino-terminal moiety (17, 18), as described for the other members of the transforming growth factor-β family of ligands (19). The antibodies used in the MIS assay do not recognize the carboxy-terminal fragment of MIS and have much lower affinity for the amino-terminal fragment than the intact protein. Although processed MIS is believed to have a fairly short circulating half-life (MacLaughlin, D. T., unpublished data), some of the individual variability in MIS concentrations might be attributed to differences in the extent of processing that has occurred *in vivo*. An assay specific for the carboxy-terminal fragment is being developed in our laboratory to resolve these issues. Further studies are also needed to assess the bioavailability of unprocessed *vs.* processed MIS.

The function subserved by the rise in testicular MIS secretion during the first year of life remains unclear. We believe that this rise may be secondary to the normal decline in gonadotropin and testosterone secretion during this same time period. Gonadotropins and testosterone decline during the latter part of the third trimester of gestation, then rise again shortly after birth to peak at 2–3 months of age, before again declining to prepubertal values by 6–7 months of age (20, 21). These changes inversely parallel the changes that occur in MIS during the first year of life. Thus, we propose that MIS expression is suppressed neonatally in conjunction with elevated levels of gonadotropins and that this suppression is released when gonadotropins return to prepubertal levels. This hypothesis is supported by *in vivo* studies demonstrating down-regulation of MIS by FSH in perinatal rodents (4, 22). Late fetal and neonatal rats treated with FSH injections had decreased expression of MIS by immunohistochemistry and Northern analysis compared to buffer-treated controls (4, 22). In these studies, testosterone was also shown to modulate MIS expression by enhancing the proteolytic processing of the full-length protein (22). Thus, the perinatal decline in testosterone levels could conceivably lead to decreased cleavage of MIS and contribute to the elevation in circulating full-length MIS soon after birth. Conversely, the surge of testosterone during early infancy could promote processing of MIS and be responsible for the gradual decline in MIS levels during late infancy. A similar process may be occurring during pubertal maturation, when testosterone levels rise and MIS levels fall. Rey and colleagues attribute the developmental changes in MIS levels to negative regulation of MIS by androgens based on the elevated MIS values found in children with androgen resistance or testosterone biosynthetic defects (23) and on the inverse relationship of MIS and testosterone (but not gonadotropins) during puberty (24). They propose that age-related differences in the sensitivity of the Sertoli cells to androgen action modulate this regulation; hence, androgen repression of MIS is less in infancy than during puberty, when a series of androgen-stimulated events culminates in maturation of the Sertoli cells and markedly decreased secretion of MIS (23, 24).

We have identified characteristics of circulating MIS levels that are helpful in several discrete clinical scenarios. The determination of circulating MIS concentrations during infancy and the early prepubertal period as a marker of Sertoli cell function in the male is useful in delineating testicular from ovarian tissue. We have found MIS determination to be invaluable in the diagnostic evaluation of infants and young children with bilateral nonpalpable gonads. In many cases, MIS levels are predictive of functional testicular tissue and correlate well with hCG stimulation testing (Lee, M. M., B. Silverman, D. T. MacLaughlin, and P. K. Donahoe, unpublished data). Furthermore, the possibility of relying on basal MIS values rather than waiting for testosterone levels after several days of hCG stimulation enables the diagnosis and management of these patients to proceed more rapidly. With the availability of this extensive normative data, particularly for infants, the determination of MIS has an undisputed role in the evaluation of patients with intersex disorders (10, 11). MIS determination is also useful as a tumor marker in women with selected types of ovarian tumors that overexpress MIS (12), both to aid with the initial diagnosis and for follow-up of tumor recurrence. The data generated in this report will enable normal and abnormal levels of MIS to be differentiated with higher precision and will facilitate the usefulness of MIS determination in the evaluation and management of various disorders of gonadal function.

Acknowledgments

We thank Drs. Michael L. Gustafson, J. H. Kim, and Elizabeth A. Catlin for help with sample collection, and Lara Asmundson for technical assistance.

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