Endocrine, *vol. 20, no. 3, 195–199, April 2003* 0969–711X/03/20:195–199/$20.00

Case Study

Assay Interference Leading to Misdiagnosis of Central Precocious Puberty

David G. Segal,[1] Linda A. DiMeglio,[1] Kenneth W. Ryder,[2] Pamela A. Vollmer,[2] and Ora Hirsch Pescovitz[1]

[1]James Whitcomb Riley Hospital for Children, Department of Pediatrics, Pediatric Endocrinology and Diabetology; and [2]Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN

Immunoassays are widely used to determine hormone levels. Antibodies directed against components of the immunoassay system can interfere with analyte concentration estimates. When unrecognized by clinicians, inappropriate clinical intervention may follow. The case of a young child with premature thelarche and elevated basal and stimulated luteinizing hormone (LH) levels is presented, in whom it is hypothesized that heterophile antibodies (HAs) caused interference in the LH immunoassay. LH concentrations were measured in two different assays: LH-microparticle enzyme immunoassay (MEIA) and LH-immunochemiluminometric assay (ICMA). To detect HA interference, LH level was remeasured after both preincubation with mouse serum to neutralize human anti-mouse antibodies, and treatment with a heterophile-blocking tube. The mean basal LH concentration by LH-MEIA was 7.4 mIU/mL and for LH-ICMA was 0.08 mIU/mL (normal range for age: 0.02–0.3 mIU/mL). LH concentration by MEIA was 0.08 mIU/mL after preincubation with mouse serum and 2.7 mIU/mL after preincubation with a heterophile blocking tube. In conclusion, HAs were identified in the serum of a child with premature thelarche. The presence of HAs led to spuriously elevated basal and gonadotropin-releasing hormone–stimulated LH concentrations, resulting in a diagnosis of central precocious puberty and unnecessary therapy. To avoid similar cases in the future, clinicians should consider the possibility of assay interference when the clinical picture is incongruent with the laboratory data.

Key Words: Precocious puberty; heterophile antibody; gonadotropin-releasing hormone.

Received October 29, 2002; Revised December 23, 2002; Accepted January 8, 2003.

Author to whom all correspondence and reprint requests should be addressed: Dr. Linda A. DiMeglio, James Whitcomb Riley Hospital for Children, 702 Barnhill Drive, Room 5960, Indianapolis, IN, 46202-5225. E-mail: dimeglio@iupui.edu

Introduction

Heterophile antibodies (HAs) are IgG antibodies to nonhuman immunoglobulins (Igs) that have been reported to cause interference in a number of clinical laboratory tests *(1–6)*. Their presence may lead to misdiagnosis or to errors in clinical management that can adversely impact patient care *(1,2,6–8)*. We present the case of a young child with premature thelarche who had basal and stimulated luteinizing hormone (LH) concentrations that were falsely elevated owing to HA interference. To our knowledge, this is the first case in which HAs in an LH assay led to an erroneous diagnosis of central precocious puberty (CPP) and unnecessary treatment.

Case Report

A 19-mo-old girl presented with Tanner stage III breast development. There was no pubic hair or acne, although there was a white vaginal discharge and estrogenized mucosa. There was no evidence of a growth spurt with height standard deviation scores (SDSs) of 2.01 and 1.76 at 10 mo and 19 mo, respectively. Bone age by examination of the left hemiskeleton was appropriate for a 19-mo-old girl (43 centers of ossification; normal range: 17.2–50.8). Magnetic resonance imaging of the head was normal. Pelvic ultrasonography revealed a prepubertal uterus (length 2.9 cm) and normal-appearing ovaries (right: 7 × 9 × 12 mm; left: 5 × 8 × 11 mm). A standard 100 μg gonadotropin-releasing hormone (GnRH) stimulation test was performed to investigate the status of the hypothalamic-pituitary-gonadal axis. A peak LH response of 16.3 mIU/mL by microparticle enzyme immunoassay (MEIA) was considered elevated. On the basis of clinical evidence of estrogen exposure and the elevated GnRH stimulated peak LH level, a diagnosis of true CPP was made and GnRH analog (GnRHa) therapy was initiated. Results of the initial and subsequent GnRH stimulation tests are presented in Table 1.

During follow-up, there was no evidence of pubertal progression, excessive linear growth, or accelerated bone age advancement, but repeat stimulation testing showed persis-

Table 1
GnRH Stimulation Tests and Treatment Time Line Indicating in Chronologic Order GnRH Stimulation Test Results and Subsequent Clinical Intervention[a]

		MEIA		ICMA	
Age	GnRH stimulation	LH (mIU/mL)	FSH (mIU/mL)	LH (mIU/mL)	FSH (mIU/mL)
		1st IRP			
19 mo	0'	9.6	5.56		
	20'	10.8	14.7		
	50'	16.3	25.8		
		Initiated GnRHa 3.5 mg q4 weekly			
25 mo	0'	19.8	0.7		
	20'	24	2.3		
	40'	19.3	2.4		
	60'	19.6	2.1		
		GnRHa increased to 7.5 mg q4 weekly			
2y 4 mo	0'	17.8	0.5		
	20'	15.4	0.7		
	40'	14.7	0.7		
	60'	13.8	0.7		
		GnRHa increased to 7.5 mg q3 weekly			
3y 8 mo	0'	16.8	0.3		
	20'	17.5	0.4		
	60'	18.6	0.5		
		Discontinued GnRHa			
		2nd IRP			
4y 8 mo	0'	37.9	0.4	0.24	0.31
	20'	38.2	0.5	0.8	0.32
	60'	38.3	0.5	0.51	0.47
5 y	0'	32.4	<0.2	<0.15	
	20'	33.0	0.7	<0.02	
	60'	28.7	0.7	<0.02	

[a]At the last time points, 4y 8mo and 5y, LH assays were run in the MEIA and ICMA assays demonstrating discordant LH levels. This raised the suspicion of HA interference. 1st IRP and 2nd IS refer to a switch in reference standards for LH that occurred in the Indiana University laboratory.

tently elevated LH levels (Table 1). In response, the dose and frequency of GnRHa injections were escalated. At 4.7 yr of age, her case was reviewed and GnRHa therapy was withdrawn. Subsequently, there was no clinical advancement in secondary sexual characteristics or bone age, but the LH elevation persisted: basal LH of 37.9 mIU/mL and peak LH of 38.3 mIU/mL. LH levels performed by an alternative immunochemiluminometric assay (ICMA) were in the prepubertal range: basal LH of 0.24 mIU/mL and peak LH of 0.8 mIU/mL. We hypothesized that HAs in the child's serum interfered with the LH immunoassays, resulting in an incorrect initial diagnosis of CPP rather than isolated premature thelarche.

Results

The LH concentrations obtained from repeat testing with both assays and other tests are presented in Fig. 1. The mean basal LH concentration by LH-MEIA was 7.4 mIU/mL (5.89, 8.3, and 8.55 mIU/mL), whereas the concentration by LH-ICMA was 0.08 mIU/mL (normal prepubertal range of 0.02–0.3 mIU/mL). The LH concentration after treatment in the heterophile blocking tube was 2.7 mIU/mL. After preincubation with mouse serum, the LH concentration by MEIA was 0.08 mIU/mL.

Discussion

HAs are common *(5)* and arise early during the generation of antibody diversity *(9–11)*. They are typically weak antibodies that are capable of recognizing a wide range of molecules *(12)*. Recently, Kaplan and Levinson *(11)* proposed a new nomenclature to classify interfering antibodies, defining HAs as endogenous antibodies that are present in the absence of a clear history of exposure to animal or other well-defined immunogens. These antibodies are multispecific in that they react with Igs from two or more species *(13)*. By contrast, human anti-animal antibodies develop in people in whom there is a clear history of treatment with animal Ig. Making this distinction is important, because both the nature of the antibodies involved and the measures one can take to circumvent them are different *(11)*.

Clinically relevant HA interference has been reported in assays for C-reactive protein (CRP) *(14)*, human chorionic gonadotropin (hCG) *(15)*, thyroid-stimulating hormone (TSH) *(7,8,16)*, luteinizing hormone (LH) *(1–3,6)*, and follicle-stimulating hormone (FSH) *(1,2,6)*. In most cases, HA interference was suspected because laboratory findings were incongruent with the clinical picture. In all cases involving gonadotropins, serum LH levels were within the normal range using alternative assay systems. Most studies confirmed HA interference by demonstrating lower or normal LH levels after preincubating the sample with animal sera. In a longitudinal observation of patients with apparent elevations in LH ($n = 15$), FSH ($n = 5$), and βhCG ($n = 9$) caused by HA interference, Dericks-Tan et al. *(6)* demonstrated temporal and quantitative differences in βhCG levels and fluctuating HA titers. We suspect that the observed temporal changes in LH-MEIA levels in our patient most likely reflect fluctuating HA titers.

Boscato and Stuart *(5)* developed a murine monoclonal antibody (MAb) assay using a modified two-site immunoradiometric assay (IRMA) to study HA interference. Using this highly sensitive assay, they found significant quantities of HA in 40% of human serum samples *(5)*. Using this model and through the investigations of others *(1–3,6–8, 14–16)*, it appears that HAs are able to cause interference in any assay system that relies upon antibody for analyte recognition. Even though HAs appear to be common, their ability to cause clinically significant interference is variable and depends on antibody concentrations, relative binding affinities, the type of assay being performed, and the assay protocol.

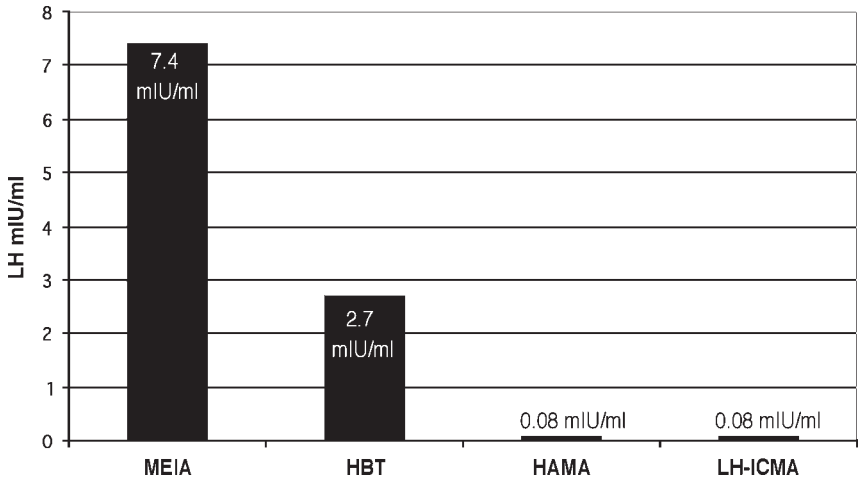

Fig. 1. Basal LH by MEIA and ICMA and investigative studies performed on single study sample. Basal LH levels were elevated in the MEIA consistent with levels in CPP. After preincubation with a heterophile blocking kit (HBT), the measured LH concentration decreased, suggesting HA interference. Preincubation with mouse serum (HAMA) neutralized the HAs that were present. The same serum assayed in the LH-ICMA yielded a prepubertal LH level.

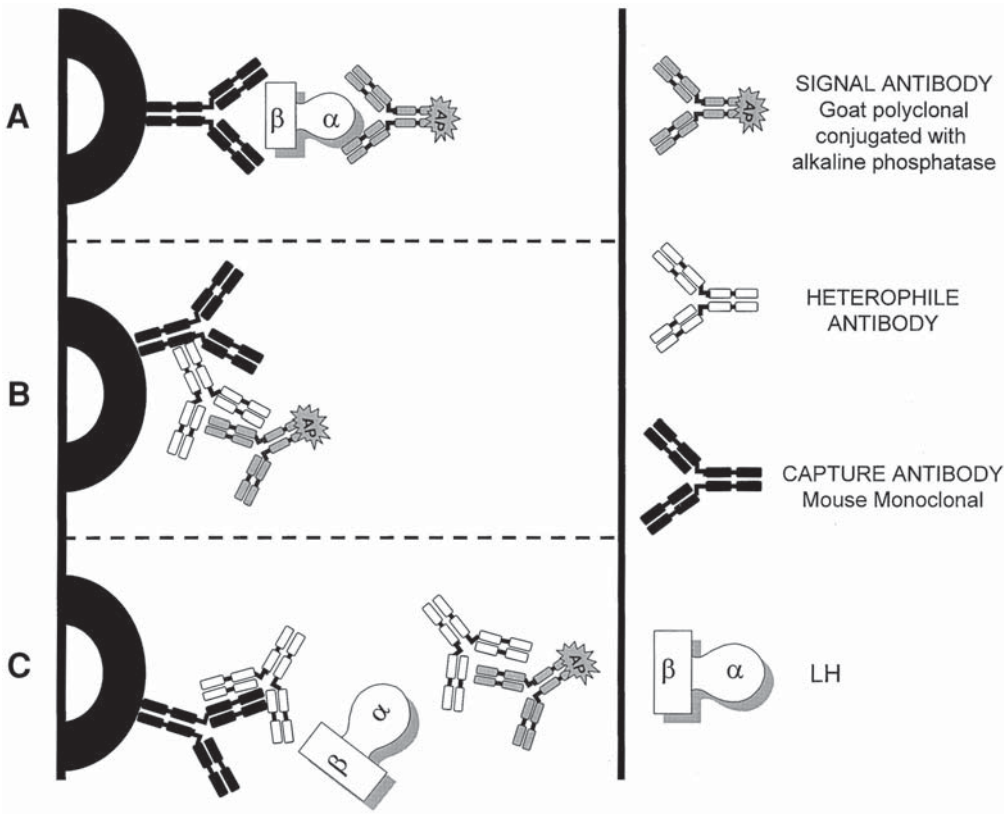

Fig. 2. Normal mechanism (**A**) and possible means (**B,C**) of HA interference in an LH-MEIA. (A) LH capture antibody is bound to a microparticle matrix. In the absence of HAs this antibody binds LH. Subsequently, a signal antibody binds the LH-capture antibody complex. (B) Interference occurs when an HA in the serum bridges the capture and the signal antibody in the absence of analyte, simulating a positive signal. This is the most likely mechanism in this case. (C) Interference occurs when an HA binds to the capture or signal antibody and inhibits analyte recognition through steric effects.

HA interference can cause spurious results in two-site immunoassays, through two probable mechanisms (Fig. 2). First, HA interference can generate positive results in the absence of analyte, by bridging the capture and signal antibody and simulating the presence of analyte (Fig. 2B). Second, when analyte is present, HAs can cause both over- and underestimates of analyte concentration in two-site immunoassays (Fig. 2C), radioimmunoassay *(1,2,8,16)*, and other

competitive binding assays when the HA interferes with analyte recognition by the reagent antibody through steric effects *(4)*. The degree of interference is dependent on the HA concentration and relative binding affinity for the reagent antibodies. Competitive binding assays are prone to HA interference because analyte is usually present in low concentrations.

HAs are relatively weak antibodies of the IgG subclass, which have been reported to bind epitopes on the Fc and Fab portions of a number of animal Igs, typically at a different site from the antigen-binding site. Similarities exist between the Fab fragments of different animal species, providing an explanation for HA crossreactivity between bovine, ovine, equine, guinea pig, rat, and monkey Igs. Mouse antibodies appear to be particularly promiscuous, whereas rabbit antibodies seem to react little with other species *(4)*.

Several measures can be taken to protect against or to minimize HA interference. These include using reagent antibodies from two different species *(5,17),* which may reduce the possibility of HA interference, but do not eliminate it. An alternative would be to use antibodies that are less prone to HA interference such as rabbit or human MAbs. It is also possible to decrease HA interference by preincubating the patient serum with serum from the same species as the reagent antibodies *(18)*, since this will precipitate the HAs. In addition, HA interference can be screened by using a commercial heterophile blocking kit that uses specific binders that inactivate HAs. If the treatment result is different from the original untreated sample result, then HA interference should be suspected.

In retrospect, our patient's initial clinical presentation was most consistent with isolated premature thelarche with lack of a growth spurt and a normal bone age. Alternative clinical management might have been to continue observation for increases in secondary sexual characteristics, evidence of bone age advancement, or increased growth velocity. An important laboratory clue to the correct diagnosis in this patient was the elevated basal LH level and the lack of a change in LH in response to GnRH agonist stimulation. This led us later to consider the possibility of assay interference. We believe that the interference was caused by HAs, as defined by Kaplan and Levinson *(11)*, because our patient had no history of exposure to goats and mice and the antibodies were multispecific, binding both goat and mouse reagent antibodies used in the LH-MEIA assay.

Conclusion

HAs were identified in the serum of a child with premature thelarche. Their presence led to spuriously elevated basal and GnRH-stimulated LH levels resulting in a diagnosis of CPP rather than isolated premature thelarche. One should focus on the clinical as well as the laboratory presentation of children with premature pubertal development. The presence of HAs should be considered when the clinical picture is incongruent with the laboratory data. The prevalence of HAs and their ability to interfere in immunoassays suggests that investigators and clinicians should be aware of their potential to impact both diagnostic and therapeutic decisions that are influenced by laboratory investigations.

Materials and Methods

The Indiana University institutional review board classified the study as "exempt," because the laboratory studies performed were part of routine patient care, of clinical relevance to the patient's care, and involved only a single patient. A single serum sample was obtained at 8 yr of age, on which all tests to requantify LH levels and to characterize the presence or absence of HAs were performed. LH levels were measured in triplicate by MEIA (Abbott Axsym system; Abbott, Chicago, IL) at the Clinical Pathology Laboratory at Indiana University, Indianapolis, IN, and once by ICMA (Esoterix; Calabasas Hills, CA) at Esoterix Endocrinology Laboratories, Calabasas, CA.

The LH-MEIA is a two-site antibody assay using a mouse monoclonal βLH capture antibody and a goat polyclonal αLH signal antibody. The assay has a lower limit of detection of 0.5 mIU/mL, using the World Health Organization (WHO). Second International Standard, Human Pituitary LH 80/522. The test is highly specific, with crossreactivities of 0.02, 0.00, and 3.5% for hCG, FSH, and TSH, respectively. At low (0–5 mIU/mL) and high (20–70 mIU/mL) LH levels, there are intraassay coefficients of variation (CVs) of 3.7 and 4.6% and interassay CVs of 6.2 and 8.4%, respectively.

The LH-ICMA is a two-site antibody assay using paired monoclonal anti-human LH antibodies, an αLH capture antibody, and a βLH signal antibody. The assay has a lower limit of detection of 0.02 mIU/mL using the WHO Second International Standard, Human Pituitary LH 80/522. The LH-ICMA exhibits minimal crossreactivity with TSH, FSH, or α-subunits. Intraassay CVs are 4.7 and 3.4%, and interassay CVs are 5.2 and 10.7% at low (0–5 mIU/mL) and high (21–70 mIU/mL) LH ranges, respectively.

In addition, the LH concentration was obtained by LH-MEIA after two additional tests. First, serum was preincubated with nonimmune mouse IgE serum (Sigma, St. Louis, MO) to neutralize human anti–mouse antibodies. Second, serum was treated with a commercially available kit to detect HA interference, Heterophile Blocking Tube, (Scantibodies, Santee, CA).

References

1. Witherspoon, L. R., Witkin, M., Shuler, S. E., Neely, H., and Gilbert S. (1986). *South Med. J.* **79(7),** 836–839.
2. Padova, G., Briguglia, G., Tita, P., Munguira, M. E., Arpi, M. L., and Pezzino, V. (1991). *Fertil. Steril.* **55(3),** 637–639.
3. Sampson, M., Ruddel, M., Zweig, M., and Elin, R. J. (1994). *Clin. Chem.* **40(10),** 1976,1977.
4. Boscato, L. M. and Stuart, M. C. (1988). *Clin. Chem.* **34(1),** 27–33.

5. Boscato, L. M. and Stuart, M. C. (1986). *Clin. Chem.* **32(8),** 1491–1495.
6. Dericks-Tan, J. S., Jost, A., Schwedes, U., and Taubert, H. D. (1984). *Klin. Wochenschr.* **62(6),** 265–273.
7. Gendrel, D., Feinstein, M. C., Grenier, J., et al. (1981). *J. Clin. Endocrinol. Metab.* **52(1),** 62–65.
8. Czernichow, P., Vandalem, J. L., and Hennen, G. (1981). *J. Clin. Endocrinol. Metab.* **53(2),** 387–393.
9. Guilbert, B., Dighiero, G., and Avrameas, S. (1982). *J. Immunol.* **128(6),** 2779–2787.
10. Levinson, S. S. (1992). *Clin. Biochem.* **25(2),** 77–87.
11. Kaplan, I. V. and Levinson, S. S. (1999). *Clin. Chem.* **45(5),** 616–618.
12. Rubin, R. L. and Theofilopoulos, A. N. (1988). *Int. Rev. Immunol.* **3(1-2),** 71–95.
13. Bouvet, J. P. and Dighiero, G. (1998). *Infect. Immun.* **66(1),** 1–4.
14. Benoist, J. F., Orbach, D., and Biou, D. (1998). *Clin. Chem.* **44(9),** 1980–1985.
15. Vladutiu, A. O., Sulewski, J. M., Pudlak, K. A., and Stull, C. G. (1982). *JAMA* **248(19),** 2489,2490.
16. Hedenborg, G., Pettersson, T., and Carlstrom, A. (1979). *Lancet* **2(8145),** 755.
17. Thompson, R. J., Jackson, A. P., and Langlois, N. (1986). *Clin. Chem.* **32(3),** 476–481.
18. Clark, P. M., Price, C. P., and Ellis, D. H. (1987). *Clin. Chem.* **33(3),** 414.