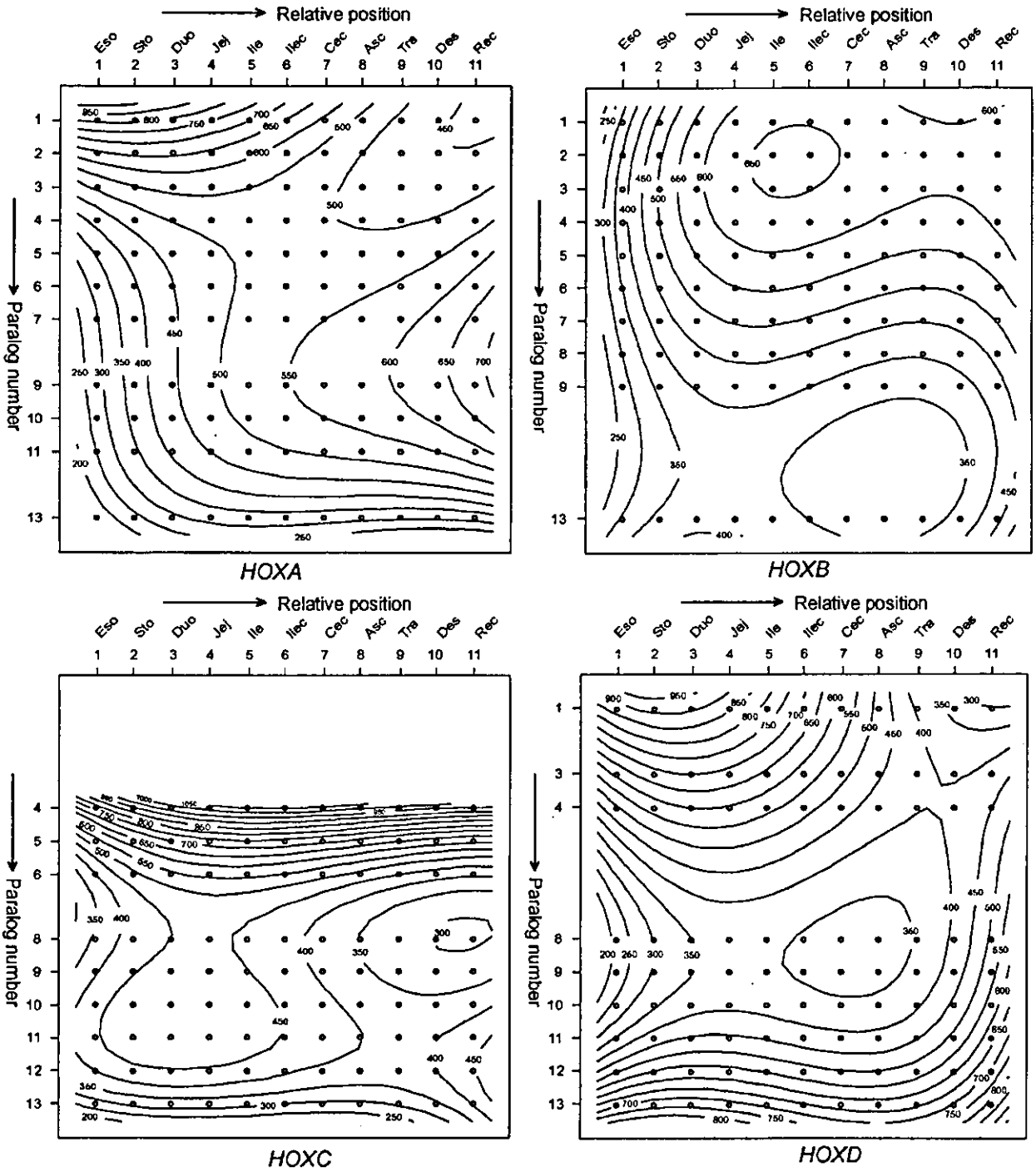

Fig. 4 Expression patterns of each *Hox* cluster gene. Three-dimensional data sets (*Hox* number, GI tract, and expression level) were fitted onto a trend-surface analysis. The *x*-axis denotes each *Hox* gene number, the *y*-axis denotes anteroposterior GI tract (esophagus = 1; stomach = 2; duodenum = 3; jejunum = 4; ileum = 5; ileocecum = 6; cecum = 7; ascending colon = 8; transverse colon = 9; descending colon = 10; and rectum = 11), and the *z*-axis denotes expression level.

sion level was low at *HOXB13*, the peak in the rectum was high. *HOXC*, *HOXC4* and *HOXC5* were extremely highly expressed, but expression gradually decreased to *HOXC6*. *HOXC8* through *HOXC11* peaked between the duodenum and jejunum, whereas *HOXC11* through *HOXC13* peaked in the rectum.

HOXD

The trend surface had two peaks, at coordinate positions (D1, 1; and D13, 11), whereas *HOXD8* and *HOXD9* were at the bottom of the *HOXD* series. Peak expression of *HOXD1* was observed in the foregut region, whereas *HOXD3* and *HOXD4* peak in the midgut region, and *HOXD8* through *HOXD13* peaked in the hindgut region (Fig. 4). Genes with a high level of expression in the foregut region tended to have a low level of expression in the hindgut region, whereas those with low levels of expression in the foregut region tended to have high levels of expression in the hindgut region.

DISCUSSION

In the present study we developed a real-time PCR-based system to simultaneously measure the expression levels of all 39 *Hox* genes. The quantitative real-time PCR method was achieved by using the intercalating dye SYBR Green, which binds to the groove of double-stranded DNA (Gibson *et al.* 1996) and emits a fluorescent signal directly that is proportional to the length of the double-stranded DNA (Morrison *et al.* 1998). Although it is simple, this detection method involves a potential source of error: the dye binds not only to the target amplification product but also to the primer dimmers, which accumulate in an unpredictable fashion (Wittwer *et al.* 1997). In the present study unwanted signals from the primer dimers were avoided by using the melting phase analysis of Ririe *et al.* (1997). This modification circumvents the problems associated with undesirable signals from the primer dimers (Higuchi *et al.* 1993).

Quantification of the target DNA by the real-time PCR method requires a highly purified control template whose molar amount is precisely known. Conventionally, the target sequence cloned into a plasmid has been used as the standard template (Gerard *et al.* 1998), and gel-purified PCR product has been used as an alternative standard template. In the present study, we employed DHPLC for purification purposes. The correlation coefficient close to 1.0 in the assay of all 39 *Hox* genes indicates an enhanced reproducibility of the system.

Hox expression profile of the esophagus, stomach, and duodenum were mapped very closely in the principal components space, and it is intriguing to note that these tissues are all derived from the foregut in the embryo. The ileum and jejunum, derived from the midgut and the descending colon and rectum, derived from the hindgut, also formed distinctive groups on the principal components space. Furthermore, expression level of *Hox* paralogs apparently reflected the paralog number and relative position along the anteroposterior axis: (1) the first three paralogs in each group are highly expressed in the foregut; (2) the *HOX7*, 9, 11, 12 paralogs were highly expressed in the cecum; (3) the *HOX*13 paralogs were expressed maximally in the rectum; and (4) the *HOX*9 paralogs were expressed maximally in the cecum and rectum (except *HOXB*).

The position-specific expression profile of *Hox* genes along the anteroposterior axis recapitulates that observed in chickens embryonic midgut and hindgut (Yokouchi *et al.* 1995b). We speculate that similarity of the embryonic and adult *Hox* expression patterns along the gut may reflect pivotal roles of *Hox* genes in the regional regenerative process of the epithelial cells, whose turnover rate is one of the fastest in adult tissues.

In conclusion, we demonstrated that *Hox* genes are expressed in a region-specific manner along the adult GI tract. Considering the critical role of *Hox* genes in morphogenesis and their abnormal expression pattern after various teratogen exposures (Jacobs *et al.* 1998; Li *et al.* 1999; Faiella *et al.* 2000), systematic profiling methods of *Hox* gene expression herein described would be a valuable tool for quantitative analysis of the homeotic potential of teratogens.

ACKNOWLEDGMENTS

This research was partially supported by a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science & Technology; the Ministry of Health, Labor, and Welfare; and Keio Gijuku Academic Development Funds.

REFERENCES

Calvo R, West J, Franklin W *et al.* (2000) Altered HOX and WNT7A expression in human lung cancer. *Proc Natl Acad Sci USA*, **97**: 12776–12781.

Cox DRH, DV (1974) *Theoretical Statistics*. Chapman & Hall, London, pp. 182–187.

Faiella A, Wernig M, Consalez GG *et al.* (2000) A mouse model for valproate teratogenicity: parental effects, homeotic transformations, and altered HOX expression. *Hum Mol Genet*, **9**: 227–236.

Favier B, Dolle P (1997) Developmental functions of mammalian Hox genes. *Mol Hum Reprod*, **3**: 115–131.

Gabriel KR (1971) The biplot graphical display of matrices with application to principal component analysis. *Biometrika*, **58**: 453–467.

Gerard CJ, Olsson K, Ramanathan R, Reading C, Hanania EG (1998) Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase

chain reaction and plasmid-DNA complementarity determining region III standards. *Cancer Res*, **58**: 3957–3964.

Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. *Genome Res*, **6**: 995–1001.

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Res*, **6**: 986–994.

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (NY)*, **11**: 1026–1030.

Hunt P, Ferretti P, Krumlauf R, Thorogood P (1995) Restoration of normal Hox code and branchial arch morphogenesis after extensive deletion of hindbrain neural crest. *Dev Biol*, **168**: 584–597.

Hunt P, Whiting J, Nonchev S *et al.* (1991) The branchial Hox code and its implications for gene regulation, patterning of the nervous system and head evolution. *Dev Suppl*, **2**: 63–77.

Jacobs HC, Bogue CW, Pinter E, Wilson CM, Warshaw JB, Gross I (1998) Fetal lung mRNA levels of Hox genes are differentially altered by maternal diabetes and butyrate in rats. *Pediatr Res*, **44**: 99–104.

Karge W, Hr Schaefer EJ, Ordovas JM (1998) Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. *Meth Mol Biol*, **110**: 43–61.

Kawazoe Y, Sekimoto T, Araki M, Takagi K, Araki K, Yamamura K (2002) Region-specific gastrointestinal Hox code during murine embryonal gut development. *Dev Growth Differ*, **44**: 77–84.

Kosaki K, Kosaki R, Suzuki T *et al.* (2002) Complete mutation analysis panel of the 39 human HOX genes. *Teratology*, **65**: 50–62.

Kosaki K, Yoshihashi H, Ohashi Y, Kosaki R, Suzuki T, Matsuo N (2001) Fluorescence-based DHPLC for allelic quantification by single-nucleotide primer extension. *J Biochem Biophys Meth*, **47**: 111–119.

Li ZL, Shiota K (1999) Stage-specific homeotic vertebral transformations in mouse fetuses induced by maternal hyperthermia during somitogenesis. *Dev Dyn*, **216**: 336–348.

Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD (1998) Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucl Acids Res*, **26**: 1396–1400.

McGinnis W, Krumlauf R (1992) Homeobox genes and axial patterning. *Cell*, **68**: 283–302.

McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring WJ (1984) A conserved DNA sequence in homoeotic genes of the Drosophila Antennapedia and bithorax complexes. *Nature*, **308**: 428–433.

Morrison TB, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*, **24**: 954–962.

Mutter G, Lea (2001) Global expression changes of constitutive and hormonally regulated genes during endometrial neoplastic transformation. *Gynecologic Oncol*, **83**: 177–185.

Pinheiro JC, Bates DM (2000) *Mixed-Effect Models in S and S-PLUS*. Springer, New York.

Pollock RA, Jay G, Bieberich CJ (1992) Altering the boundaries of Hox3.1 expression: evidence for antipodal gene regulation. *Cell*, **71**: 911–923.

Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*, **245**: 154–160.

Roberts DJ, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C (1995) Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development*, **121**: 3163–3174.

Roberts DJ, Smith DM, Goff DJ, Tabin CJ (1998) Epithelial-mesenchymal signaling during the regionalization of the chick gut. *Development*, **125**: 2791–2801.

Scott MP (1992) Vertebrate homeobox gene nomenclature. *Cell*, **71**: 551–553.

Steuerwald N, Cohen J, Herrera RJ, Brenner CA (2000) Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol Hum Reprod*, **6**: 448–453.

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques*, **22**: 130–138.

Yokouchi Y, Nakazato S, Yamamoto M *et al.* (1995a) Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev*, **9**: 2509–2522.

Yokouchi Y, Sakiyama J, Kuroiwa A (1995b) Coordinated expression of Abd-B subfamily genes of the HoxA cluster in the developing digestive tract of chick embryo. *Dev Biol*, **169**: 76–89.

Yokouchi Y, Sasaki H, Kuroiwa A (1991) Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature*, **353**: 443–445.

Eur J Pediatr (2004) 163: 745–746
DOI 10.1007/s00431-004-1556-0

SHORT REPORT

Goro Sasaki · Tomohiro Ishii · Seiji Sato
Ken Hoshino · Yasuhide Morikawa · Hiroko Kodama
Nobutake Matsuo · Takao Takahashi
Tomonobu Hasegawa

Multiple polypoid masses in the gastrointestinal tract in patient with Menkes disease on copper-histidinate therapy

Received: 8 April 2004 / Accepted: 7 September 2004 / Published online: 7 October 2004

We describe a Japanese boy with Menkes disease who developed multiple gastrointestinal polypoid masses on the palate, the posterior wall in the oral part of the pharynx, the gastric body, and pyloric antrum, during copper-histidinate therapy restoring normal serum copper levels.

A Japanese boy was born as the first child of an unrelated couple after an uncomplicated pregnancy. He was vaginally delivered at 38 weeks' gestation with a birth weight of 2,520 g, a length of 48.5 cm, and a head circumference of 33.0 cm. At birth, no abnormal physical findings were recorded. He was referred to our service because of hypotonia at 7 months of age. His weight was 6,755 g (3rd percentile), length 66.1 cm (10th–25th percentile), and head circumference 43.3 cm (25th percentile). Physical examination showed bronze and steely hair and moderate hypotonia. Neither head control nor eye contact was complete. The diagnosis of Menkes disease was made based on the following findings: a serum copper level of 13 μg/dl (reference range 80–160 μg/l), coeruloplasmin 5 mg/dl (reference range 21–53 mg/dl), tortuous cerebral vessels on a brain MRI scan and a diverticulum in the bladder on ultrasonography. The *ATP7A* gene was successfully amplified by PCR using patient's DNA from exons 1 to 6, but not from exons 7 to 23, whereas all the exons of the *ATP7A* gene were amplified using control DNA (data not shown). This led to the genetic diagnosis of a large deletion encompassing the part of the *ATP7A* gene which was reported previously in Menkes disease [1]. Subcutaneous copper histidinate therapy (90 μg/kg per day) has been introduced, which might lead to neurological improvement [3]. During therapy, the serum copper level was restored to normal (82–97 μg/dl) with some improvement in muscle tone and spontaneous motor activity. At 15 months of age, he presented with bloody vomiting. On endoscopy, a mildly hypertrophic mass was found in the pyloric antrum which was thought to be the cause of the bleeding. Later he had intermittent bloody vomiting. Repeated endoscopy showed an increase in the size of the polypoid mass in the pyroric antrum (up to 4 cm in diameter) which obstructed the antrum. Other polypoid masses were seen on the palate, posterior wall in the oral part of the pharynx, and the gastric body (Fig. 1). The polypoid masses on the palate were somewhat hard and movable on palpation. The pharyngeal lesions appeared soft on endoscopy. The lesions on the pyloric antrum and gastric body looked clearly polypoid. Histological examination of the polymoid masses in the pyloric antrum showed a hyperplastic polyp, foveolar cell type. A biopsy of other lesions was not obtained. Because of feeding difficulties, bloody vomiting, and poor general condition, colonostomy was performed at the age of 29 months.

Menkes disease is an X-linked recessive disorder of the copper membrane transport system due to mutations in the *ATP7A* gene (OMIM 309400) and characterised by neurodegeneration in early infancy, failure to thrive, and connective tissue abnormalities such as bladder diverticula [1]. A solitary polypoid mass was reported in three cases of Menkes disease, all of which were found in the pyloric antrum [2,5]. Polypoid masses may develop anywhere in the gastrointestinal tract and can be revealed by endoscopy, as in our case.

G. Sasaki · T. Ishii · S. Sato · N. Matsuo · T. Takahashi
T. Hasegawa (✉)
Department of Paediatrics,
Keio University School of Medicine,
35 Shinanomachi Shinjuku-ku,
160-8582 Tokyo, Japan
E-mail: thaseg@sc.itc.keio.ac.jp
Tel.: +81-3-33531211 ext 62365
Fax: +81-3-53791978

K. Hoshino · Y. Morikawa
Department of Surgery,
Keio University School of Medicine,
Tokyo, Japan

H. Kodama
Department of Paediatrics,
Teikyo University School of Medicine,
Tokyo, Japan

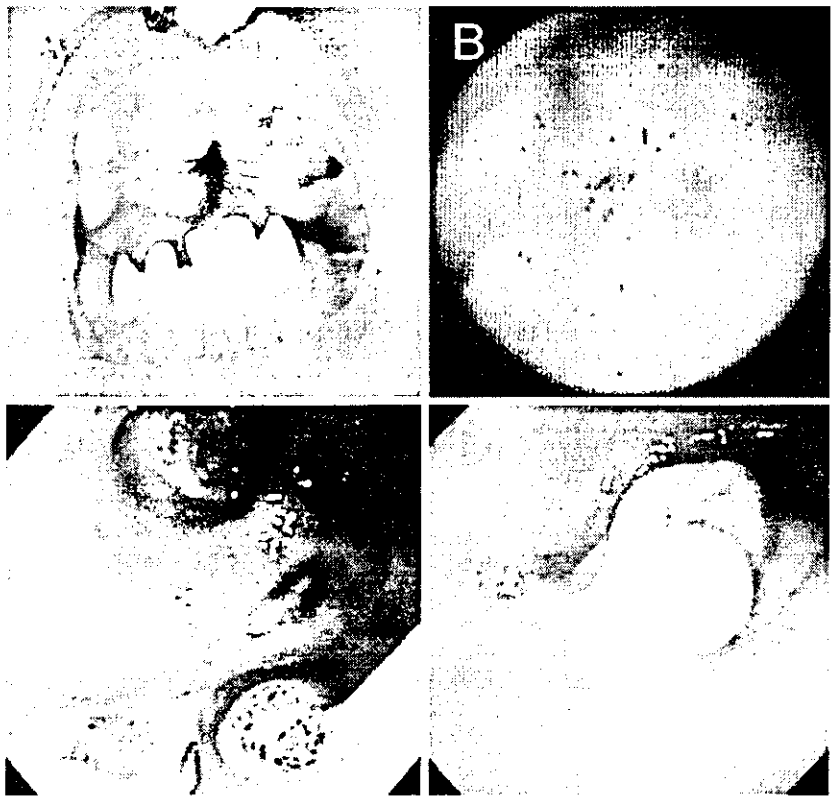

Fig. 1 Multiple polypoid masses in the gastrointestinal tract in the patient at the age of 22 to 29 months. **A** Palate. **B** Posterior wall in the oral part of the pharynx (oral indicates the oral side of the pharynx). **C** Gastric body. The gastric lesions showed evidence of haemorrhage. **D** Pyloric antrum

The failure to prevent the development of polypoid masses by appropriate copper-histidinate therapy can be explained as follows. The obligatory connective tissue weakness in Menkes disease can predispose to mucosal abnormalities anywhere within the gastrointestinal tract, with subsequent development of polypoid masses. The connective tissue weakness is believed to be due to low lysyl oxidase activity. Lysyl oxidase is the copper-dependent enzyme responsible for oxidative deamination of lysine and hydroxylysine as the first step in collagen cross-link formation [4]. Within the Golgi body, this enzyme is synthesised and binds to copper being transported by ATP7A. In Menkes disease, defective ATP7A causes a low copper content in the Golgi body leading to low enzymatic activity of lysyl oxidase. Copper-histidinate therapy, theoretically, cannot restore the copper content in the Golgi body due to defective ATP7A.

Acknowledgements This publication was supported by the Pfizer Fund for Growth and Development.

References

1. Culotta VC, Gitlin JD (2001) Menkes disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 3115–3118
2. Kaler SG, Westman JA, Bernes SM, Elsayed AM, Bowe CM, Freeman KLB, Wu CD, Wallach MT (1993) Gastrointestinal hemorrhage associated with gastric polyps in Menkes disease. J Pediatr 122: 93–95
3. Kreuder J, Otten A, Fuder H, Tumer Z, Tonnensen T, Horn N, Dralle D (1993) Clinical and biochemical consequences of copper-histidine therapy in Menkes disease. Eur J Pediatr 152: 828–832
4. Siegel RC (1979) Lysyl oxidase. Int Rev Connective Tiss Res 8: 73–118
5. Wheeler EM, Roberts PJ (1975) Menkes's steely hair syndrome. Arch Dis Child 5: 269–274

Endocrine Journal 2003, **50** (6), 783–792

Reference Values for Urinary Steroids in Japanese Newborn Infants: Gas Chromatography/Mass Spectrometry in Selected Ion Monitoring

KEIKO HOMMA, TOMONOBU HASEGAWA*, MASAMI MASUMOTO, EIKO TAKESHITA, KIYOAKI WATANABE, HITOSHI CHIBA**, TAKAO KUROSAWA***, TAKAO TAKAHASHI* AND NOBUTAKE MATSUO*,#

Department of Laboratory Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
**Department of Pediatrics, Keio University School of Medicine, Tokyo 160-8582, Japan*
***Department of Laboratory Medicine, Hokkaido University, Sapporo 060-8648, Japan*
****Faculty of Pharmaceutical Sciences, Health Science University of Hokkaido, Hokkaido 061-0293, Japan*
#National Center for Child Health and Development, Tokyo 157-8535, Japan

Abstract. Urinary steroid profile analysis using gas chromatography/mass spectrometry (GC/MS) has been reported for the diagnosis of abnormal steroidogenesis in newborn infants with some success. We tried to establish the reference values of 63 urinary steroids in Japanese newborn infant, using GC/MS in selected ion monitoring (SIM) that utilizes two characteristic mass ions for each steroid for definitive identification. We studied 36 healthy full-term newborn infants (1–56 days of age) on spot urine samples to define the reference values (mg/g creatinine, median and 10–90 percentile range) and to investigate the possible difference between daytime and nighttime levels. We also studied 23 healthy adult females (20–24 years of age) on 24-hour-urine for the comparison of the reference values of newborn infants. Fifty metabolites of DHEA, pregnenolone, 17-hydroxypregnenolone, androstenedione, progesterone, 17-hydroxyprogesterone, 21-deoxycortisone, corticosterone, 18-hydroxycorticosterone, aldosterone, 18-hydroxycortisol, 11-deoxycortisol, cortisone, cortisol, and estrogen in each infant were measurable without interference, but 13 metabolites of 11-hydroxyandrostenedione, pregnenolone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, 21-deoxycortisol, 11-deoxycortisol and cortisol were unmeasurable in each infant due to the interference of fetal cortex steroids as confirmed by abnormal peak area ratios of two mass ions. All 63 metabolites in each control adult were measurable without interference. 16α-, 16β-, and 15β-hydroxy metabolites of 3β-hydroxy-5-en-steroids, and 6β-, 18-hydroxy and 11-oxo-metabolites of corticosteroids were significantly higher in full-term newborn infants than those in adults as previously reported. Urinary steroids showed little circadian variation in the newborn infants, indicating that spot urine can substitute for 24-hour urine.

Key words: Steroidogenesis, Urinary steroids, Profile analysis, GC/MS-SIM, Newborn infant, Reference values
(*Endocrine Journal* **50**: 783–792, 2003)

URINARY steroid profile analysis using gas chromatography/mass spectrometry (GC/MS) has been reported to be useful in the diagnosis of abnormal steroidogenesis in newborn infants and in adults by various authors [1–3]. However, the risk of interference in the determination of the selected steroid always exists even in the most sensitive and selective GC/MS in selected ion monitoring (SIM) method. To avoid false positive errors caused by a large quantity of fetal cortex steroids in the urine samples of newborn infants, we measured each steroid by two characteristic mass ion monitoring rather than multiple single-ion-monitoring method developed by Shackleton [1].

The reference values of urinary steroids in newborn infants using GCMS-SIM have been recently reported by Caulfield *et al.* (spot urine samples, μg/g creati-

Received: March 12, 2003
Accepted: August 15, 2003
Correspondence to: Keiko HOMMA, Department of Laboratory Medicine, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan

nine) [2], Shackleton (24 hr urine samples, μg/10 mg creatinine) [4], and Malunowicz *et al.* (24 hr urine samples, μg/day) [3], but never in Japanese newborn infants. Nor is there any previous study on circadian variation of urinary steroids in full-term newborn infants.

The purpose of the present study is 1) to try to determine the reference values (mg/g creatinine, median and 10–90 percentile range) of 63 urinary steroids (44 steroids previously reported as the diagnostic markers and other 19 minor metabolites) in the spot urine samples of newborn infants, 2) to compare the reference values of newborn infants and adults, and 3) to study the possible circadian variation of urinary steroids in full-term newborn infants.

Materials and Methods

Fifty-five reference steroids and 3 internal standards were purchased from Sigma-Aldorich (St. Louis, MO, U.S.A.) or Steraloids Inc. (Newport, RI, U.S.A.), and 18-hydroxycortisol was synthesized by us (H.C. and T.K.) [5]. Derivatizing reagents and enzymes were obtained as described below. 1-Trimethylsilyl imidazol (TSIM) was obtained from Shin-Etsu Chemical (Tokyo, Japan), o-methylhydroxyamine hydrochloride from Wako Pure Chemistries (Osaka, Japan), N-methyl-N-trimethyltrifluoro acetamide (MSTFA) from Macherey-Nagel GmbH (Düren, Germany). Glufatase kit type II, which consists of the reagent for hydrolysis containing 4200 Fishman U of β-glucuronidase and 2100 Roy U of sulfatase originated from *Ampullaria* digestive juice and the reagents for removal of phosphates, was purchased from Nippon Biotest (Tokyo, Japan).

Daytime spot urine samples were obtained from 18 full-term newborn infants (12 males and 6 females, 5–22 days of age) to set forth the reference values of various urinary steroids. A total of 46 spot urine samples were obtained from another 18 full-term newborn infants (thirteen 1-8-day-old infants and ten 25-56-day-old infants) in the daytime (0600 h–1400 h) and at night (2100 h–2400 h) to investigate the possibility of circadian variation. Twenty-four-hr urine samples were obtained from 23 adult females of 20–24 years of age at various menstrual cycles. Each sample was kept at –20°C until analyzed. Each subject gave their informed consent.

Urine steroid profile analysis was performed according to methods previously reported [2, 3, 6–10]. In brief, the method consisted of enzymatic hydrolysis of 0.5–2 ml of urine sample, extraction, derivative formation, purification, GC/MS-SIM analysis and quantification. The procedures were as follows: 1) chemical removal of inhibitors of enzymes such as phosphatic and sulphatic anions before hydrolysis, 2) two hr hydrolysis at 60°C by *Ampullaria* enzymes, 3) extraction of the freed steroids by dichloromethane from urine saturated with sodium sulfate anhydrous, 4) addition of three internal standards (5 μg/sample) and drying under nitrogen, 5) o-methyloxime-trimethylsilyl derivatization carried out by reaction with 100 μl of 2% o-methylhydroxyamine hydrochloride solution in pyridine for 1 hr at 52°C, followed by trimethylsilylation with 100 μl TSIM for 17 hr at 75°C, 6) purification of derivatives by dichloromethane, 0.5% H_2SO_4 and distilled water, followed by drying under nitrogen, 7) dissolution of derivatives in 40 μl MSTFA, and 8) GC/MS-SIM analysis with two characteristic mass ions selected for each steroid (Table 1). GC/MS-SIM analysis was performed on a HP 5890II GC with a HP fused silica column (25 m) coupled to a HP 5971 MS (Agilent Technologies, Palo Alto, CA, U.S.A.). Temperature programming was 140°C (1 min), 140–180°C (30°C/min), 180–280°C (2°C/min) and 280°C (20 min). The ratio of first ion peak area of each steroid to that of stigmasterol (one of the internal standards) was used for quantification. The calibration curve was calculated from three mixed standard solutions (200, 400, 1000 ng/5 ml).

The reference values (10, 50, 90 percentile) were determined using percentile analysis. Statistical differences between groups were analyzed by Mann-Whitney U test or Wilcoxon signed rank test.

Results

1) Basic properties of the method

The procedures were completed within 32 hr. The extraction rates were 93 +/– 10% (average +/– s.d.), and the recovery rates from urine were 103 +/– 12% (Table 2). The sensitivity was 5–20 pg/injection recognized in dihydroxypregnenolone, trihydroxypregnene, dihydroxypregnanolone, trihydroxypregnanolone and trihydroxypregnane, but was 500–1000 pg/injection

in 3-oxo metabolites of cortisol and corticosterone (Table 2). The interassay C.V. (coefficient variation) of 56 steroids (equivalent to 10 ng/injection) was 7 +/– 4 % (n = 52) and the interassay C.V. and intra-assay C.V. of 25 steroids (>0.1 mg/g creatinine) in pooled urine was 11 +/– 6 % (n = 37) and 4 +/– 4% (n = 8).

2) Measurable urinary steroids

The urinary steroids measurable by this method are shown in Fig. 1. Fifty metabolites of DHEA, pregnenolone, 17-hydroxypregnenolone, androstenedione, progesterone, 17-hydroxyprogesterone, 21-deoxycortisone, corticosterone, 18-hydroxycorticosterone, aldosterone, 18-hydroxycortisol, 11-deoxycortisol, cortisone, cortisol, and estrogen in each infant were measurable without interference, but 13 metabolites of 11-hydroxyandrostenedione, pregnenolone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, 21-deoxycortisol, 11-deoxycortisol and cortisol were unmeasurable due to interference of fetal cortex steroids as confirmed by abnormal peak area ratios of two mass ions. All 63 metabolites in each control adult were measurable without interference.

3) Reference values

The reference values (10, 50, 90 percentile) in 18 full-term newborn infants and 23 adult females are shown in Table 3. The significant differences between newborn infants and adults were recognized in 41 steroids (metabolites of androstenedione, DHEA, pregnenolone, progesterone, aldosterone, 11-deoxycortisol, cortisone, and cortisol) by Mann-Whitney U test and shown with "s" in Table 3.

4) Circadian variation

There were no significant differences in main metabolites of DHEA, pregnenolone, 17OHP, and cortisone between daytime urine (0600 h–1400 h) and night urine (2100 h–2400 h) of infants aged 1 week (1–8 days) or 1 month (25–56 days) by Wilcoxon signed rank test (Table 4).

Discussion

We successfully established the reference values of 50 urinary steroids in full-term newborn infants by GC/MS-SIM. The median values (in mg/g creatinine) of 16αHD, 16βHD, 16oxoAD5, AT5, 16HP5, 21HP5, 1517DHP5, PT5, 5β17HP, 20αPT, Ptl, 1517DHP, 5βTHE, 20αCtl, and 20βCtl in our newborn subjects approximated the mean values (in mg/g creatinine) of these steroids reported by Caulfield *et al.* [2] and Shackleton *et al.* [4].

We failed to determine the reference values of 13 steroids including the main metabolites of deoxycorticosterone, corticosterone and cortisol (THDOC, 5βTHA, 5αTHA, 5βTHB, 5αTHB, 5β THF and 5αTHF). The failure is due to a large amount of fetal cortex steroids that precluded the measurement of the selected steroids since no valid method has been established to separate fetal cortex steroids from the main metabolites of corticosterone and cortisol [4]. We could not measure other important 6α-hydroxy metabolites of corticosteroids (6α-hydroxyTHE, 6α-hydroxy-Ctl, 6α-hydroxy-THA, etc. [11, 12]) due to lack of standards. We could, however, estimate the secretion levels of corticosterone and cortisol depending on the excretion of 6β-hydroxycorticosterone, 5βTHE, 5αTHE, 20αCtl, 20βCtl, 20αDHE, 20βDHE, E and F.

Twenty-six steroid metabolites were significantly higher in full-term newborn infants than those in adults as previously reported [13–16], and 15 steroid metabolites were significantly lower in full-term newborn infants than those in adults. Little difference was found between male and female adults except for the metabolites of progesterone, 17OHP and estrogen (data not shown). Increased amounts of metabolites of pregnenolone and DHEA in newborn infants are of fetal cortex origin, with fetal cortex expressing higher P450scc and P450c17 and lower 3β-hydroxysteroid dehydrogenase activities [17–20]. Increased amounts of 16α-hydroxy metabolites of pregnenolone and DHEA and of 6β-hydroxy metabolite of corticosterone in newborn infants were due to higher P4503A7 activity in the fetal liver. P4503A7 has higher 16α- and 6β-hydroxylation activities [21–27]. Increased amounts of 11-oxo metabolites and decreased amounts of 11β-hydroxy metabolites of cortisol in newborn infants are due to higher 11β-hydroxysteroid dehydrogenase type II activity in fetal kidney, skin, and colon [28, 29]. Other metabolites such as metabolites of

Table 1. The essentials of the GC/MS-SIM analysis

Serum steroids						Urinary steroids				
No. in Fig. 1	Abbreviation	name	No. in Fig. 1	Abbreviation	name (*previously reported for diagnosis)	1st ion (m/e)	2nd ion (m/e)	retention time (min)	Ion ratio (%)	calibrated by
1	P5	5-pregnene-3β-ol-20-one	2	PD5	5-pregnene-3β,20α-diol	372.2	462.3	48.3	86	itself
			3	16HP5	5-pregnene-3β,16α-diol-20-one	* 474.3	384.2	51.8	21	itself
			4	16PT5	5-pregnene-3β,16α,20β-triol	* 460.3	445.3	53.2	31	itself
			5	21HP5	5-pregnene-3β,21-diol-20-one	* 505.4	474.3	53.6	70	itself
6	P4	4-pregnene-3,20-dione	7	PD	5β-pregnane-3α,20α-diol	* 269.1	284.1	46.1	112	itself
8	DOC	4-pregnene-21-ol-3,20-dione	9	THDOC	5β-pregnane-3α,21-diol-20-one	* 507.4	476.3	49.5	186	itself
10	B	4-pregnene-11β,21-diol-3,20-dione	11	5βTHB	5β-pregnane-3α,11β,21-triol-20-one	* 564.4	474.3	53.3	228	itself
			12	5αTHB	5α-pregnane-3α,11β,21-triol-20-one	* 564.4	474.3/595.4	53.9	220, 50	itself
			13	20βDHB	4-pregnene-11β,20β,21-triol-3-one	537.4	357.2	66.0	14	itself
			14	6HB	4-pregnene-6β,11β,21-triol-3,20-dione	621.4	469.3	(63.0) 63.7	—	itself
15	A	4-pregnene-21-ol-3,11,20-trione	16	5βTHA	5β-pregnane-3α,21-diol-11,20-dione	* 431.3	521.4/490.3	52.8	89, 183	itself
			17	5αTHA	5α-pregnane-3α,21-diol-11,20-dione	* 490.3	521.4	54.0	35	itself
18	18HB	4-pregnene-11b,18,21-triol-3,20-dione	19	18HTHA	5β-pregnane-3α,18,21-triol-11,20-dione	* 457.3	488.3	55.7, 58.8	10, 38	THAld
20	Ald	4-pregnene-11β,21-diol-3,18,20-trione	20	Ald	4-pregnene-11β,21-diol-3,18,20-trione	459.3	(531.4)	(59.7) 59.9 (61.5) (61.8)	—	itself
			21	THAld	5β-pregnane-3α,11β,21-triol-18,20-dione	* 506.4	(490.3)	53.0, 55.3	—	itself
22	17HP5	5-pregnene-3β,17α- diol-20-one	22	17HP5	5-pregnene-3β,17α-diol-20-one	474.3	505.4	49.8	51	itself
			23	PT5	5-pregnene-3β,17α,20β-triol	* 433.3	343.2	52.3	38	itself
			24	5,16Adien	5,16-androstadiene-3β-ol	344.2	215.1	27.4	152	itself
			25	15H17HP5	5-pregnene-3β,15β,17α-triol-20-one	* 258.1	593.4	50.3	8	20αP4
26	17HP4	4-pregnene-17α-ol-3,20-dione	27	5β17HP	5β-pregnane-3α,17α-diol-20-one	* 476.3	364.2	43.6	43	itself
			28	5α17HP	5α-pregnane-3α,17α-diol-20-one	* 476.3	364.2	44.6	41	itself
			29	20βPT	5β-pregnane-3α,17α,20β-triol	435.3	255.1	46.0	195	itself
			30	20αPT	5β-pregnane-3α,17α,20α-triol	* 435.3	255.1	47.1	205	itself
			31	5α20αPT	5α-pregnane-3α,17α,20α-triol	435.3	255.1	47.5	179	20αPT
			32	15H17HP	5β-pregnane-3α,15β,17α-triol-20-one	* 258.1	564.4	44.4	35	20αP4
33	21DOF	4-pregnene-11β,17α-diol-3,20-dione	34	11H17HP	5β-pregnane-3α,11β,17α-triol-20-one	564.4	474.2	48.0	50	itself
			35	20αP4	5β-pregnane-3α,11β,17α,20α-tetrol	523.4	253.1	51.2	24	itself
36	21DOE	4-pregnene-17α-ol-3,11,20-trione	37	11o17HP	5β-pregnane-3α,17α-diol-11,20-dione	490.3	521.4	47.3	35	itself
			38	Ptl	5β-pregnane-3α,17α,20α-triol-11-one	* 449.3	359.2	50.9	58	itself
39	S	4-pregnene-17α,21-diol-3,20-dione	40	5βTHS	5β-pregnane-3α,17α,21-triol-20-one	* 564.4	474.3	49.0	42	itself
			41	5αTHS	5α-pregnane-3α,17α,21-triol-20-one	564.4	474.3	50.3	53	itself
42	F	4-pregnene-11β,17α,21-triol-3,20-dione	42	F	4-pregnene-11β,17α,21-triol-3,20-dione	* 515.4	361.2	(64.2) 64.6	47	itself
			43	6HF	4-pregnene-6β,11β,17α,21-tetrol-3,20-dione	* 513.4	384.2	(65.2) 66.2	116	itself
			44	5βTHF	5β-pregnane-3α,11β,17α,21-tetrol-20-one	* 472.3	562.4	54.2	83	itself
			45	5αTHF	5α-pregnane-3α,11β,17α,21-tetrol-20-one	* 472.3	562.4	54.5	81	itself
			46	20αCor	5β-pregnane-3α,11β,17α,20α,21-pentaol	* 343.2	523.4	57.9	48	itself

Table 1. (continued)

Serum steroids						Urinary steroids				
No. in Fig. 1	Abbreviation	name	No. in Fig. 1	Abbreviation	name (*previously reported for diagnosis)	1st ion (m/e)	2nd ion (m/e)	retention time (min)	Ion ratio (%)	calibrated by
			47	20βCor	5β-pregnane-3α,11β,17α,20β,21-pentaol	* 343.2	523.4	56.3	38	itself
			48	20αDHF	4-pregnene-11β,17α,20α,21-tetrol-3-one	* 476.3	578.4	(68.5) 69.2	31	20βDHF
			49	20βDHF	4-pregnene-11β,17α,20β,21-tetrol-3-one	* 476.3	578.4	(66.0) 66.7	32	itself
50	E	4-pregnene-17α,21-diol-3,11,20-trione	50	E	4-pregnene-17α,21-diol-3,11,20-trione	* 531.4	562.4	(61.1) 61.7	58	itself
			51	5βTHE	5β-pregnane-3α,17α,21-triol-11,20-dione	* 488.3	578.4	52.4	123	itself
			52	5αTHE	5α-pregnane-3α,17α,21-triol-11,20-dione	488.3	578.4	54.2	70	itself
			53	20αCtl	5β-pregnane-3α,17α,20α,21-tetrol-11-one	* 449.3	551.4	55.3	7	itself
			54	20βCtl	5β-pregnane-3α,17α,20β,21-tetrol-11-one	* 449.3	551.4	56.4	13	itself
			55	5α20βCtl	5α-pregnane-3α,17α,20β,21-tetrol-11-one	449.3	551.4	56.8	6	20βCtl
			56	20αDHE	4-pregnene-17α,20α,21-triol-3,11-dione	402.3	—	(64.4) 65.3	—	20βDHE
			57	20βDHE	4-pregnene-17α,20β,21-triol-3,11-dione	402.3	(488.3)	(65.8) 66.8	—	itself
58	18HF	4-pregnene-11β,17α,18,21-tetrol-3,20-dione	58	18HF	4-pregnene-11β,17α,18,21-tetrol-3,20-dione	* 385.2	412.3	70.5, 76.2, 76.6	20, 14, 26	itself
59	DHEA	5-androstene-3β-ol-17-one	59	DHEA	5-androstene-3β-ol-17-one	* 260.1	358.2	40.0	93	itself
			60	AD5	5-androstene-3β,17β-diol	* 344.2	434.3	40.4	44	itself
			61	16αHD	5-androstene-3β,16α-diol-17-one	* 446.3	477.3	44.8 (45.1)	10	itself
			62	16βHD	5-androstene-3β,16β-diol-17-one	446.3	462.3	46.9, 47.1	5	itself
			63	16oAD5	5-androstene-3β,17α-diol-16-one	* 446.3	462.3	48.0	36	itself
			64	AT5	5-androstene-3β,16α,17α-triol	* 432.3	522.4	48.6	22	itself
65	AD	4-androstene-3,17-dione	66	An	5α-androstane-3α-ol-17-one	* 270.1	360.2	37.1	72	itself
			67	Et	5β-androstane-3α-ol-17-one	* 270.1	360.2	37.6	61	itself
68	11HAD	4-androstene-11β-ol-3,17-dione	69	11HA	5α-androstane-3α,11β-diol-17-one	* 358.2	448.3	43.6	175	itself
			70	11HE	5β-androstane-3α,11β-diol-17-one	* 358.2	448.3	44.0	278	itself
71	11oAD	4-androstene-3,11,17-trione	72	11OA	5α-androstane-3α-ol-11,17-dione	* 374.2	405.3	40.8	28	itself
			73	11OE	5β-androstane-3α-ol-11,17-dione	* 315.2	405.3	40.9	61	itself
74	El	estrane-3-ol-17-one	74	El	estrane-3-ol-17-one	371.2	340.2	40.9	69	itself
75	E2	estrane-3,17β-diol	75	E2	estrane-3,17β-diol	416.3	285.1	41.8	84	itself
76	E3	estrane-3,16α,17β-triol	76	E3	estrane-3,16α,17β-triol	* 504.4	311.2	49.7	115	itself
		Internal Standard 1		5αCho	5α-cholestane	262.1	372.2	47.3	358	itself
		Internal Standard 2		Stig	stigmasterol	394.2	484.3	64.4	74	itself
		Internal Standard 3		C.B.	cholesterol butylate	368.2	353.2	73.1	20	itself

Abbreviations: "No. in Fig. 1" = Sequential number of serum and urinary steroids in Fig. 1 (map). "1st ion" = the 1st characteristic mass ion for calculation. "2nd ion" = the 2nd characteristic mass ion for monitoring interferences.

Identification: The steroid identification in each sample is based on (1) the ratio of second mass ion to first mass ion and (2) the difference between the retention time of each steroid and 5α-cholestane. The second ion number between parentheses in Ald, THAld, and 20βDHE was the characteristic mass ion of E, 20βCtl, and 20βDHF.

Calibration: Each of the 56 steroids was calibrated by its respective reference standard. Seven steroids (15,17-DHP5, 5α20bPT, 15,17-DHP, 18HTHA, 5α20βCtl, 20αDHE, and 20αDHF) were calibrated by other reference standards (20αP4, 20αPT, 20αP4, THAld, 20βCtl, 20βDHE, and 20βDHF). A major peak area was used for calculation in each of the 59 steroids, but a plural peak area was used for 16βHD, THAld, 18HTHA, and 18HF. The retention times of the sub peaks not used for calculation are indicated in parentheses.

Table 2. Basic properties of the GCMS-SIM analysis

No. in Fig. 1	Steroid name	Extraction rate	Recovery rate	Sensitivity	Reproducibility			
		Standard (equivalent to 10 ng/injection) added to water (%)	standard (equivalent to 10 ng/injection) added to pooled urine (%)	(pg/injection)	standard (10 ng/injection)	pooled urine		
					Interassay n = 52	interassay n = 37		intraassay n = 8
					C.V. (%)	average (mg/g creatinine)	C.V. (%)	C.V. (%)
2	PD5	94	117	20	5	0.066	34	42
3	16HP5	96	91	10	8	0.034	13	2
4	16PT5	92	80	5	3	0.042	14	2
5	21HP5	96	117	20	3	0.094	11	2
7	PD	86	93	20	4	0.152	7	3
9	THDOC	91	90	100	4	0.016	29	8
11	5βTHB	96	103	100	4	0.097	11	2
12	5αTHB	96	104	50	4	0.309	7	3
13	20βDHB	99	88	500	20	0.000	0	0
14	6HB	110	90	500	12	0.036	0	8
16	5βTHA	96	91	50	4	0.143	8	1
17	5αTHA	92	84	50	5	0.113	9	2
19	18HTHA	—	—	—	—	0.081	36	4
20	Ald	89	87	100	10	0.044	0	21
21	THAld	64	106	50	6	0.039	22	7
22	17HP5	92	107	10	3	0.005	27	10
23	PT5	90	106	5	5	0.268	8	3
24	5,16Adien	72	112	20	7	0.048	21	6
25	15H17HP5	—	—	—	—	0.000	0	0
27	5β17HP	89	111	5	4	0.307	6	3
28	5α17HP	90	94	5	4	0.035	8	2
29	20βPT	86	111	20	8	0.013	22	3
30	20αPT	83	110	5	14	0.616	8	15
31	5α20αPT	—	—	—	—	0.029	13	16
32	15H17HP	—	—	—	—	0.000	0	0
34	11H17HP	92	101	20	4	0.016	0	23
35	20αP4	94	96	20	5	0.023	11	3
37	11o17HP	105	113	20	7	0.007	23	0
38	Ptl	99	89	10	4	0.014	10	6
40	5βTHS	87	114	20	9	0.053	16	2
41	5αTHS	84	112	50	6	0.003	42	14
42	F	94	100	1000	11	0.073	21	7
43	6HF	62	115	1000	16	0.249	20	5
44	5βTHF	88	117	100	6	1.279	8	4
45	5αTHF	93	83	50	6	1.266	9	3
46	20αCor	89	102	50	9	0.314	11	1
47	20βCor	90	122	50	14	0.166	13	2
48	20αDHF	—	—	—	—	0.079	16	2
49	20βDHF	99	88	500	11	0.075	16	2
50	E	85	78	500	8	0.116	11	2
51	5βTHE	80	100	20	5	2.115	8	3
52	5αTHE	80	94	20	6	0.167	8	2
53	20αCtl	99	133	20	5	0.845	7	2
54	20βCtl	93	96	20	10	0.574	10	16

Table 2. (continued)

No. in Fig. 1	Steroid name	Extraction rate	Recovery rate	Sensitivity	Reproducibility			
		Standard (equivalent to 10 ng/injection) added to water (%)	standard (equivalent to 10 ng/injection) added to pooled urine (%)	(pg/injection)	standard (10 ng/injection)	pooled urine		
					Interassay n = 52	interassay n = 37		intraassay n = 8
					C.V. (%)	average (mg/g creatinine)	C.V. (%)	C.V. (%)
55	5α20βCtl	—	—	—	—	0.080	13	15
56	20αDHE	—	—	—	—	0.100	14	2
57	20βDHE	106	96	50	10	0.040	23	2
58	18HF	88	109	100	17	0.163	16	2
59	DHEA	100	94	5	5	0.846	15	3
60	AD5	90	109	5	5	0.126	7	4
61	16αHD	96	106	10	4	0.362	19	3
62	16βHD	61	92	20	11	0.084	19	7
63	16αAD5	113	107	100	4	0.055	16	32
64	AT5	90	104	5	4	0.199	16	8
66	An	96	104	10	5	1.752	9	3
67	Et	95	104	5	4	0.929	8	3
69	11HA	97	106	20	5	0.444	6	3
70	11HE	97	109	20	4	0.038	11	5
72	11OA	95	117	20	5	0.025	23	8
73	11OE	99	113	20	4	0.075	11	3
74	E1	108	114	10	8	0.012	47	5
75	E2	94	118	5	4	0.001	41	0
76	E3	91	106	10	4	0.005	17	9

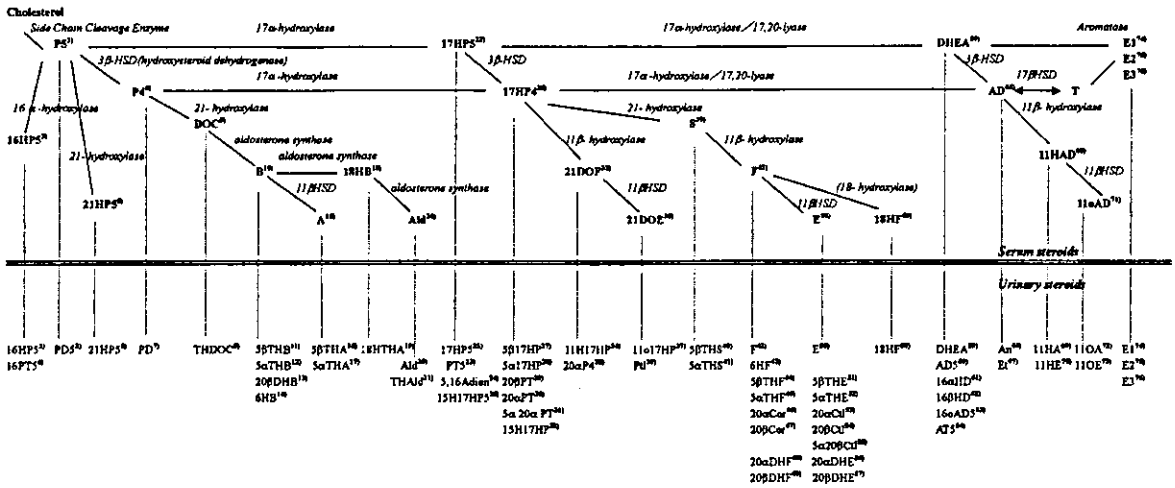

Fig. 1. Steroid metabolism map

aldosterone, 18-hydroxycortisol, and 16β- and 15β-hydroxy metabolites, remain unexplained for the difference between newborn infants and adults.

There is no previous study on circadian variation of urinary steroids expressed in mg/g creatinine in full-term newborn infants as measured by GC/MS-SIM. Our study disclosed little circadian variation in urinary steroids in 1-week-old infants and 1-month-old

Table 3. Reference values of urinary steroids (mg/g creatinine) in Japanese control full-term newborn infants and adult females

No.in Fig. 1	Steroid name	M-W U test	Infants (M = 12, F = 6) 5–22 days of age			Adults (F = 23) 20–24 years of age		
			10% tile	50% tile	90% tile	10% tile	50% tile	90% tile
2	PD5					0.010	0.032	0.059
3	16HP5	s	2.121	9.784	53.112	0.005	0.017	0.048
4	16PT5	s	0.014	0.080	0.200	0.004	0.010	0.054
5	21HP5	s	2.294	3.870	8.515	0.056	0.087	0.127
7	PD	s	0.031	0.069	0.210	0.286	0.478	4.548
9	THDOC					0.004	0.007	0.024
11	5βTHB					0.052	0.077	0.128
12	5αTHB					0.114	0.159	0.233
13	20βDHB	s	0.000	0.000	0.153	0.000	0.048	0.208
14	6HB	s	0.161	1.477	3.991	0.000	0.000	0.019
16	5βTHA					0.072	0.098	0.173
17	5αTHA					0.035	0.047	0.086
19	18HTHA	s	0.045	0.144	0.681	0.022	0.032	0.055
20	Ald	s	0.000	0.372	0.791	0.008	0.012	0.032
21	THAld	s	0.159	0.366	1.030	0.014	0.027	0.058
22	17HP5	s	0.004	0.029	0.273	0.001	0.003	0.016
23	PT5	s	0.037	0.094	0.267	0.139	0.233	0.510
24	5,16Adien	s	0.101	0.188	0.466	0.032	0.058	0.090
25	15H17HP5	s	1.310	4.367	9.703	0.000	0.000	0.000
27	5β17HP	n	0.026	0.124	0.207	0.060	0.141	0.619
28	5α17HP	n	0.000	0.026	0.054	0.005	0.012	0.049
29	20βPT	n	0.000	0.019	0.089	0.005	0.016	0.053
30	20αPT	s	0.057	0.104	0.217	0.288	0.495	1.029
31	5α20αPT	n	0.000	0.006	0.032	0.007	0.012	0.032
32	15H17HP	s	0.027	0.108	0.236	0.000	0.000	0.009
34	11H17HP	s	0.000	0.000	0.000	0.000	0.001	0.003
35	20αP4	n	0.000	0.000	0.033	0.000	0.007	0.021
37	11o17HP	s	0.000	0.000	0.020	0.000	0.000	0.004
38	Ptl	s	0.000	0.004	0.020	0.004	0.008	0.013
40	5βTHS	s	0.055	0.235	0.548	0.028	0.061	0.098
41	5αTHS					0.000	0.004	0.012
42	F	n	0.000	0.000	0.866	0.045	0.075	0.097
43	6HF					0.093	0.178	0.300
44	5βTHF					1.008	1.397	1.982
45	5αTHF					0.510	0.920	1.485
46	20αCor					0.236	0.337	0.461
47	20βCor					0.312	0.523	0.728
48	20αDHF	s	0.000	0.000	0.000	0.074	0.103	0.188
49	20βDHF	s	0.000	0.000	0.000	0.066	0.081	0.159
50	E	s	0.629	1.286	2.374	0.072	0.110	0.153
51	5βTHE	s	3.642	5.723	11.364	1.734	2.569	3.734
52	5αTHE	s	0.086	0.272	1.504	0.063	0.082	0.174
53	20αCtl	s	0.065	0.229	0.862	0.762	0.942	1.448
54	20βCtl	s	0.697	1.773	3.639	0.388	0.542	0.760
55	5α20βCtl	s	0.011	0.025	0.156	0.039	0.048	0.085
56	20αDHE	s	0.108	0.674	2.263	0.063	0.085	0.143
57	20βDHE	s	0.199	0.513	2.073	0.024	0.031	0.046
58	18HF	s	0.461	0.897	1.970	0.060	0.099	0.154
59	DHEA	s	0.000	0.059	0.320	0.053	0.237	2.181
60	AD5	s	0.000	0.014	0.032	0.010	0.044	0.277

Table 3. (continued)

No.in Fig. 1	Steroid name	M-W U test	Infants (M = 12, F = 6) 5–22 days of age			Adults (F = 23) 20–24 years of age		
			10% tile	50% tile	90% tile	10% tile	50% tile	90% tile
61	16αHD	s	1.329	8.056	53.066	0.120	0.364	0.741
62	16βHD	s	3.229	8.994	32.770	0.010	0.029	0.062
63	16oAD5	s	0.614	5.055	20.759	0.024	0.046	0.081
64	AT5	s	1.221	6.260	24.680	0.111	0.293	0.460
66	An	s	0.018	0.064	0.233	0.636	1.213	1.897
67	Et	s	0.005	0.009	0.027	0.613	1.001	1.549
69	11HA	s	0.026	0.110	0.233	0.345	0.508	0.865
70	11HE					0.028	0.052	0.424
72	11OA	s	0.037	0.082	0.141	0.009	0.019	0.076
73	11OE	s	0.081	0.139	0.273	0.063	0.107	0.428
74	E1	n	0.000	0.016	0.034	0.006	0.015	0.033
75	E2	n	0.000	0.008	0.028	0.001	0.002	0.009
76	E3	n	0.000	0.009	0.034	0.002	0.009	0.024

Abbreviations: "M-W U test" = Mann-Whitney U test. "s" = significantly different ($p<0.05$). "n" = not significantly different. "blank" = measurement interfered with unknown substances

Table 4. Comparison of urinary steroids obtained at daytime and at night in thirteen 1-week-old infants and ten 1-month-old infants

No.	Steroid	Average (mg/g creatinine) in 1-week-old infants (1–8 days of age, n = 13)		Wilcoxon signed rank test	Average (mg/g creatinine) in 1-month-old infants (25–56 days of age, n = 10)		Wilcoxon signed rank test
		Daytime (0600 h–1400 h)	Night (2200 h–2400 h)		Daytime (1300 h–1500 h)	Night (2100 h–2200 h)	
9	16αHD	15.63	16.80	n	15.22	10.11	n
15	16HP5	18.98	19.28	n	9.92	7.53	n
26	20αPT	0.12	0.20	n	0.26	0.23	n
29	Ptl	0.00	0.00	n	0.02	0.01	s ($p<0.05$)
47	5βTHE	5.30	5.38	n	15.87	12.83	n

Abbreviations: "s" = significantly different ($p<0.05$). "n" = not significantly different.

infants, indicating that spot urine substitutes for 24-hour urine. Unfortunately, we have not compared spot urine and 24-hour urine in given subjects.

Our reference values of urinary steroids are relevant to the laboratory diagnosis of various adrenal disorders in newborn infants. We are currently addressing the clinical utility of our method in differential diagnosis of inborn error of steroidogenesis in newborn infants.

Acknowledgements

We wish to thank Drs. Hisami Iri and Teruko Uchida (Keio University) for fruitful discussions and Dr. Makoto Ueki of Mitsubishi Kagaku Bio-Clinical Labs for his kind suggestions for improving our paper. We also thank Dr. Masamine Saito of Tsuzuki Medical Clininc for critical comments. This work was supported in part by The Pfizer Fund for Growth and Development.

References

1. Shackleton CHL (1993) Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. *J Steroid Biochem Molec Biol* 45: 127–140.
2. Caulfield MP, Lynn T, Gottschalk ME, Jones KL, Taylor NF, Malunowicz EM, Shackleton CHL, Reitz RE, Fisher DA (2002) The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens. *J Clin Endocrinol Metab* 87: 3682–3690.
3. Malunowicz EM, Mitkowska Z, Bal K, Nizankowska-

Blaz T, Moszczynska E, Iwanicka Z, Romer TE (1997) Definitive diagnosis of enzymatic deficiencies of steroidgenesis in at-risk newborns and infants by urinary marker analysis using GCMS-SIM. *Horm Res* 48: 243–251.
4. Shackleton CHL, Honour JW, Taylor NF (1979) Metabolism of fetal and neonatal adrenal steroids. *J Steroid Biochem* 11: 523–539.
5. Kurosawa T, Ikegawa S, Chiba H, Ito Y, Nakagawa S, Kobayashi K, Tohma M (1992) Convenient synthesis of 18-hydroxylated cortisol and prednisolone. *Steroids* 57: 426–429.
6. Palermo M, Gomez-Sanchez C, Roitman E, Shackleton CHL (1997) Quantitation of cortisol and related 3-oxo-4-ene steroids in urine using gas chromatography/mass spectrometry with stable isotope-labeled internal standards. *Steroids* 61: 583–589.
7. Steen G, Tas AC, Ten Noever de Brauw MC, Drayer NM, Wlothers BG (1980) The early recognition of the 21-hydroxylase deficiency variety of congenital adrenal hyperplasia. *Clin Chim Acta* 105: 213–224.
8. Axelson M, Sahlberg BL, Sjovall J (1981) Analysis of profiles of conjugated steroids in urine by ion-exchange separation and gas chromatography-mass spectrometry. *J Chromatogr* 224: 355–370.
9. Vrbanac JJ, Sweeley CC, Pinkton JD (1983) Automated metabolic profiling analysis of urinary steroids by a gas chromatography mass spectrometry data system. *Biomed Mass Spectrom* 10: 155–161.
10. Pike AW, Moynihan C, Kibler S, Marsh GP, Fennessey PV (1984) Semi-micro quantitative analysis of complex urinary steroid mixtures in healthy and diseased states. *J Chromatogr* 306: 39–50.
11. Taylor NF, Curnow DH, Shackleton CHL (1978) Analysis of glucocorticoid metabolites in the neonatal period: Catabolism of cortisone acetate by an infant with 21-hydroxylase deficiency. *Clin Chim Acta* 85: 219–229.
12. Derks HJGM, Drayer NM (1978) The identification and quantification of three new 6α-hydroxylated corticosteroids in human neonatal urine. *Steroids* 31: 289–305.
13. Mitchell FL, Shackleton CHL (1969) The investigation of steroid metabolism in early infancy. *Adv Chim Chem* 12: 141–215.
14. Shackleton CHL, Taylor NF (1975) Identification of the androstenetriolones and androstenetetrols present in the urine of infants. *J Steroid Biochem* 6: 1393–1399.
15. Wallace AM, Beesley J, Thomson M, Giles CA, Ross AM, Taylor NF (1987) Adrenal status during the first month of life in mature and premature human infants. *J Endocrinol* 112: 473–480.
16. Taylor N, Sharma S, Giffin F, Naik S, Greenough A (1996) Urinary excretion of adrenocortical steroid metabolites by pre-term human infants: Trends from birth until 3 months post term. *Endocr Res* 22: 741–749.
17. Lack EE, Kozakewich HPW (1990) Embryology, developmental anatomy, and selected aspects of non-neoplastic pathology. In: Lack EE (ed) Pathology of the Adrenal Glands. Churchill Livingstone Inc. Contemporary Issues in Surgical Pathology Vol.14. 1–74.
18. Narasaka T, Suzuki T, Moriya T, Sasano H (2001) Temporal and spatial distribution of corticosteroidogenic enzymes immunoreactivity in developing human adrenal. *Mol Cell Endocrinol* 174: 111–120.
19. Coulter CL, Goldsmith PC, Mesiano S, Voytek CC, Martin MC, Mason JI, Jaffe RB (1996) Functional maturation of the primate fetal adrenal in vivo II. Ontogeny of corticosteroid synthesis is dependent upon specific zonal expression of 3β-hydroxysteroid dehydrogenase/isomerase. *Endocrinology* 137: 4653–4659.
20. Coulter CL, Jaffe RB (1998) Functional maturation of the primate fetal adrenal in vivo: III. Specific zonal localization and developmental regulation of CYP21A2 (P450c21) and CYP11B1/CYP11B2 (P450c11/aldosterone synthase) lead to integrated concept of zonal and temporal steroid biosynthesis. *Endocrinology* 139: 5144–5150.
21. de Wildt SN, Kearns GL, Leeder JS, van den Anker JN (1999) Cytochrome P450 3A: Ontogeny and drug disposition. *Clin Pharmacokinet* 37: 485–505.
22. Ohmori S, Fujiki N, Nakasa H, Nakamura H, Ishii I, Itahashi K, Kitada M (1998) Steroid hydroxylation by human fetal CYP3A7 and human NADPH-cytochrome P450 reductase coexpressed in insect cells using baculovirus. *Res Commun Mol Pathol Pharmacol* 100: 15–28.
23. Tateishi T, Nakura H, Asoh M, Watanabe M, Tanaka M, Kumai T, Takashima S, Imaoka S, Funae Y, Yabusaki Y, Kamataki T, Kobayashi S (1977) A comparison of hepatic cytochrome P450 protein expression between infancy and postinfancy. *Life Sci* 61: 2567–2574.
24. Schuetz JD, Beach DL, Guzelian PS (1994) Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics* 4: 11–20.
25. Rane A, Henningsson S, Ask B, Ladona MG (1992) Comparison of human fetal hepatic and adrenal cytochrome P450 activities with some major gestational steroids and ethylmorphine as substrates. *J Steroid Biochem Mol Biol* 43: 335–341.
26. Maenpaa J, Pelkonen O, Cresteil T, Rane A (1993) The role of cytochrome P450 3A (CYP3A) isoform(s) in oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *J Steroid Biochem Mol Biol* 44: 61–67.
27. Vauzelle-Kervroedan F, Rey E, Pariente-Khayat A, Bienvenu T, Badoual J, Olive G, Pons G (1996) Non invasive in vivo study of the maturation of CYP IIIA in neonates and infants. *Eur J Clin Pharmacol* 51: 69–72.
28. Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, Stewart PM (1998) Expression of type 2 11β-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. *J Clin Endocrinol Metab* 83: 4490–4497.
29. Hirasawa G, Sasano H, Suzuki T, Takeyama J, Muramatu Y, Fukushima K, Hiwatashi N, Toyota T, Nagura H, Krozowski ZS (1999) 11β-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor in human fetal development. *J Clin Endocrinol Metab* 84: 1453–1458.

ORIGINAL ARTICLE

Coronary risk factors in Kawasaki disease treated with additional gammaglobulin

M Miura, H Ohki, T Tsuchihashi, H Yamagishi, Y Katada, K Yamada, Y Yamashita, A Sugaya, O Komiyama, H Shiro

Arch Dis Child 2004;**89**:776–780. doi: 10.1136/adc.2003.032748

See end of article for authors' affiliations

Correspondence to: Dr M Miura, Keio-Northwestern Clinic, 1701 East Woodfield Rd., #140, Schaumburg, IL 60173, USA; miura@as.email.ne.jp

Accepted 7 October 2003

Aims: To assess the hypothesis that an additional intravenous gammaglobulin (IVGG) infusion, if administered early, may prevent coronary artery lesions (CAL) in patients with Kawasaki disease (KD) who do not respond to initial IVGG therapy.
Methods: Forty four KD patients (17 with CAL and 27 without CAL), treated with additional IVGG because of persistent or recrudescent fever after initial IVGG therapy, were studied. Main outcome measures were the presence of CAL by echocardiography and the number of febrile days before and after start of additional IVGG infusion (pre- and post-additional IVGG).
Results: In univariate analyses, risk factors for CAL were the number of febrile days pre-additional IVGG, the number of febrile days post-additional IVGG, the number of days that initial IVGG was divided over, the white blood cell count pre- and post-additional IVGG, and the C reactive protein concentration pre-additional IVGG. In a multivariate analysis, the only independent risk factor was the number of febrile days pre-additional IVGG (≥10 days; odds ratio 7.86; 95% CI 1.44 to 42.8; p=0.02).
Conclusions: Among KD patients with persistent or recrudescent fever after initial IVGG therapy, administration of additional IVGG before the first 10 febrile days was associated with a decreased prevalence of CAL, when compared with the prevalence in those who were retreated later. An additional IVGG infusion, if administered early, may prevent CAL in initial IVGG non-responders.

Approximately 10–20% of patients with acute Kawasaki disease (KD) do not respond to standard intravenous gammaglobulin (IVGG) therapy and have persistent or recrudescent fever after completion of the infusion.[1–10] Additional IVGG therapy is commonly used for such patients,[1–4] but a preventive effect on the development of coronary artery lesions (CAL) has not been proved by retrospective studies[1–3] making a comparison between KD patients treated with additional IVGG and those not treated with additional IVGG. According to the study by Burns and colleagues,[1] among patients with persistent or recrudescent fever after initial IVGG therapy, CAL developed in five of 25 patients (20%) treated with additional IVGG and none of 21 patients (0%) not treated with additional IVGG. In the studies by Han and colleagues[2] and Durongpisitkul and colleagues,[3] the prevalence of CAL tended to be higher in KD patients who received additional IVGG than in those who did not receive additional IVGG (43% *v* 27% and 21% *v* 9%, respectively). Other reports[4–6] have also shown that the prevalence of CAL is high (33–54%) in patients treated with additional IVGG. This is probably because physicians are inclined to give additional IVGG to patients who appeared more ill and were believed to be at high risk for CAL.[1] Furthermore, we suspect that a delayed start of additional IVGG may fail to prevent CAL, since prolonged fever is a powerful predictor of CAL;[11] however, few previous studies[1 4] have referred to the number of febrile days before start of the additional IVGG infusion. We therefore focused on KD patients treated with additional IVGG owing to unresponsiveness to initial IVGG, and studied the risk factors for CAL to test a hypothesis that additional IVGG therapy, if administered early, may decrease the prevalence of CAL.

METHODS

Subjects

We reviewed the medical records of all the patients with acute typical KD at our 12 institutions between January 1993 and December 1999. Patients who received additional IVGG therapy within 14 days after completion of the initial IVGG infusion were selected for the present study. We excluded patients treated with corticosteroids, ulinastatin (a urine protease inhibitor),[12] or both combined with additional IVGG to eliminate possible confounding effects. Although the additional therapies were indicated for patients who had persistent or recrudescent fever after completion of initial IVGG therapy, the decision on the administration was at the discretion of the treating physician. Serial echocardiograms were performed at admission, and one or two times a week until the 30th illness day.

Data

Medical record review was performed to extract demographic data, the course of fever, treatments received, laboratory data, and echocardiographic findings. Fever was defined as body temperature of 38.0°C or higher,[4 9 10] and the number of febrile days was calculated for periods pre-initial, pre-additional, and post-additional IVGG therapies. The periods both pre-initial and pre-additional IVGG covered days from the onset of fever to the starting day of each IVGG infusion. The period post-additional IVGG covered the day following the starting day of the additional IVGG infusion to the day 14 days later. If patients had recrudescent fever, we counted only febrile days excluding afebrile days. For both initial and additional IVGG, we analysed the number of illness days at the start of infusion, the dose per body weight, and the number of days that the dose was divided over. For laboratory data, we assessed both the white blood cell count and the C reactive protein concentration pre-initial, pre-additional, and post-additional IVGG therapies. We designated the data both pre-initial and pre-additional IVGG as those taken on either the starting day of each IVGG infusion

Abbreviations: CAL, coronary artery lesions; IVGG, intravenous gammaglobulin; KD, Kawasaki disease

or the previous day. We designated the data post-additional IVGG as those taken during a period between the 2nd day and the 5th day after the starting day of the additional IVGG infusion. When we obtained laboratory data two or more times during these periods, we adopted the later data for pre-initial and pre-additional IVGG and the earlier data for post-additional IVGG.

The subjects were divided into two groups according to the presence or absence of CAL determined by echocardiography after about one month of illness, or 30±5 illness days. CAL were defined as the internal lumen diameter greater than two standard deviations above the expected mean calculated for body surface area on the basis of the study by De Zorzi and colleagues.[13]

Statistics

The relation between the variables considered as possible risk factors and the presence of CAL was examined by univariate logistic regression analyses. Continuous variables were transformed into categorical variables by the cut-off points that were defined using receiver operating characteristic curves. Six months of age was also used for the cut-off point of age.[14 15] Variables found to be associated with the presence of CAL in the univariate analyses were subjected to multivariate logistic regression analyses. All analyses were performed using SPSS, version 11.0 (SPSS Inc., Chicago, IL).

RESULTS

Of 816 patients with acute typical KD in our 12 hospitals in Japan, 746 received initial IVGG (1–2 g/kg) administration plus oral aspirin (30–50 mg/kg/day). Brands used for IVGG were freeze dried sulphonated product (Venilon), polyethylene glycol treated product (Venoglobulin IH or Glovenin I), and PH4 treated acidic product (Polyglobin N). A total of 81 (10.9%) patients were treated with additional therapies because of persistent fever (n = 65) or recrudescent fever (n = 16) after completion of the initial IVGG infusion. The fractions of the patients were not significantly different in any factors of initial IVGG infusion such as the number of illness days at the start, the dose, and the number of days that the dose was divided over (table 1). For additional therapies, 48 patients were treated with IVGG (0.5–2 g/kg), 24 with IVGG combined with corticosteroids (1–2 mg/kg/day), ulinastatin (15 000 U/kg/day), or both, and nine with corticosteroids or ulinastatin. Furthermore, a third course of treatment was given to nine patients: IVGG (n = 1), IVGG with ulinastatin (n = 5), corticosteroids (n = 1), and corticosteroids with ulinastatin (n = 2). Overall, we gave additional IVGG without corticosteroids or ulinastatin to 46 patients, two of whom were excluded from the study because they received it more than 14 days after completion of the infusion of initial IVGG. Of 44 patients who met the inclusion criteria, 17 (38.6%) had CAL.

In univariate analyses, the significant risk factors for CAL ($p < 0.05$) were the number of febrile days pre-additional IVGG (≥10 days), the number of febrile days post-additional IVGG (≥2 days), the number of days that initial IVGG was divided over (4 or 5 days), the white blood cell count pre-additional IVGG (≥16 000/μl), the C reactive protein concentration pre-additional IVGG (≥9.7 mg/dl), and the white blood cell count post-additional IVGG (≥11 300/μl) (table 2). Data regarding the white blood cell count and the C reactive protein concentration post-additional IVGG were unavailable in five patients, all of whom had no CAL. The number of illness days at the start of initial IVGG infusion was same as the number of febrile days pre-initial IVGG in all patients. The number of illness days at the start of additional IVGG infusion (≥10 days) was less significantly associated with CAL ($p = 0.008$) in comparison with the number of febrile days pre-additional IVGG ($p = 0.002$); hence, the number of febrile days pre-additional IVGG, rather than the number of illness days at the start of additional IVGG infusion, was subjected to multivariate analyses.

We performed a multiple logistic regression analysis for the variables significantly associated with the risk for CAL, excluding or including the white blood cell count post-additional IVGG separately because of unavailable data (table 3). In both exclusion and inclusion of the white blood cell count post-additional IVGG, the only independent risk factor was the number of febrile days pre-additional IVGG (≥10 days).

Of 17 patients with CAL, none was found to have CAL before the start of the initial IVGG infusion. CAL were detected in 14 patients (13 males and one female; age range 1–91 months) after start of the initial IVGG infusion before start of the additional IVGG infusion. Among the 14 patients, additional IVGG was given to three before 10 febrile days and to 11 on or after 10 febrile days. CAL were detected in three patients (a 2 month old female, an 8 month old male, and a 13 month old male), after the start of the additional IVGG infusion. Additional IVGG was given to all of the three patients on or after 10 febrile days.

No adverse events were observed in any patient treated with additional IVGG. There were no signs of hypotension, congestive heart failure, allergic reaction, or hyperviscosity.

DISCUSSION

In the present study, the number of febrile days pre-additional IVGG (≥10 days) was an independent risk factor for CAL in patients with acute KD who did not respond to initial IVGG therapy and received additional IVGG therapy. It is possible that additional IVGG starting before the first 10 febrile days may prevent CAL. In this connection, let us recall the well known recommendation[7 11] that initial IVGG should be given within the first 10 illness days. We believe that "febrile days", rather than "illness days", may be a better marker of the need for additional IVGG in recrudescent fever, on the basis of the result that the presence of CAL was more significantly related to the number of febrile days pre-additional IVGG than the illness days at the start of the infusion of additional IVGG. Few previous reports regarding additional IVGG therapy[3–6] did not refer to the timing of administration of additional IVGG; the high reported prevalence of CAL may reflect a delayed start. Prolonged fever for 10 days or longer as a risk factor for CAL is supported by the following data. (1) In a pathological study,[16] the formation of CAL began after 10 illness days. (2) In an echocardiographic study,[17] CAL appeared at around 10 illness days. (3) In a clinical study,[11] patients with CAL had a significantly higher temperature than those without CAL during 10–13 illness days. Since additional IVGG appeared to be safe without apparent adverse effects,[4] we suggest that it should be administered before the first 10 febrile days to initial IVGG non-responders.

Our results indicated that age, sex, white blood cell count, and C reactive protein concentration were not independent risk factors for CAL. On the other hand, age less than 6 months[14 15] and male sex[15] were reported to be risk factors for CAL in KD patients treated with IVGG. It has also been reported that CAL were associated with abnormal laboratory data pre-initial IVGG, including high counts of neutrophils and bands,[1 10] and abnormal laboratory data post-initial IVGG, including increased white blood cell count and increased C reactive protein concentration.[14 15] This may be attributed to subject differences: the present study concerned only patients treated with additional IVGG, while previous studies[3 10 14 15 18 19] concerned all KD patients treated with IVGG. There is a possibility that additional IVGG reduced the

Table 1 Univariate analysis of fractions of patients treated with additional therapies in initial IVGG therapy

	No. of patients treated with initial IVGG	No. of patients treated with additional therapies (%)	Odds ratio (95% CI)	p value
No. of illness days at the start of infusion				
<5 days	226	29 (13)		
≥5 days	520	52 (10)	0.91 (0.77 to 1.07)	0.25
Dose				
<1.5 g/kg	152	11 (7)		
≥1.5 g/kg	594	70 (12)	1.20 (0.96 to 1.49)	0.11
No. of days that the dose was divided over				
1 or 2 days	236	33 (14)		
4 or 5 days	510	48 (9)	0.86 (0.74 to 1.01)	0.06

CI, confidence interval; IVGG, intravenous gammaglobulin.

Table 2 Univariate analysis of risk factors for coronary artery lesions

Risk factor	No. of patients	No. of patients with CAL (%)	Odds ratio (95% CI)	p value
Age				
<6 months	9	5 (56)		
≥6 months	35	12 (34)	0.25 (0.09 to 1.85)	0.25
<22 months	25	11 (44)		
≥22 months	19	6 (32)	0.59 (0.17 to 2.05)	0.40
Sex				
Male	33	15 (45)		
Female	11	2 (18)	0.29 (0.05 to 1.43)	0.12
Febrile days pre-initial IVGG				
<5 days	14	5 (36)		
≥5 days	30	12 (40)	0.83 (0.22 to 3.11)	0.79
Febrile days pre-additional IVGG				
<10 days	22	3 (14)		
≥10 days	22	14 (64)	11.1 (2.48 to 49.5)	0.002
Febrile days post-additional IVGG				
<2 days	28	6 (21)		
≥2 days	16	11 (69)	8.06 (2.01 to 32.3)	0.003
Dose of initial IVGG				
<1.5 g/kg	4	2 (50)		
≥1.5 g/kg	40	15 (38)	0.60 (0.08 to 4.72)	0.63
No. of days that initial IVGG was divided over				
1 or 2 days	16	2 (13)		
4 or 5 days	28	15 (54)	8.06 (1.17 to 41.7)	0.01
Dose of additional IVGG				
<1.5 g/kg	13	4 (31)		
≥1.5 g/kg	31	13 (42)	1.62 (0.41 to 6.45)	0.69
No. of days that additional IVGG was divided over				
1 or 2 days	26	8 (31)		
4 or 5 days	18	9 (50)	2.25 (0.65 to 7.81)	0.20
White blood cell count pre-initial IVGG				
<11 300/μl	17	5 (29)		
≥11 300/μl	27	12 (44)	1.92 (0.53 to 6.98)	0.32
CRP pre-initial IVGG				
<12.2 mg/dl	24	9 (38)		
≥12.2 mg/dl	20	8 (40)	1.11 (0.33 to 3.78)	0.87
White blood cell count pre-additional IVGG				
<16 000/μl	24	4 (17)		
≥16 000/μl	20	13 (65)	9.28 (2.26 to 38.1)	0.002
C reactive protein pre-additional IVGG				
<9.7 mg/dl	26	6 (23)		
≥9.7 mg/dl	18	11 (61)	5.24 (1.41 to 19.6)	0.01
White blood cell count post-additional IVGG*				
<11 300/μl	22	6 (27)		
≥11 300/μl	17	11 (65)	4.88 (1.25 to 19.2)	0.02
C reactive protein post-additional IVGG*				
<5.2 mg/dl	22	7 (32)		
≥5.2 mg/dl	17	10 (59)	3.06 (0.82 to 11.4)	0.10

*Data not available for five patients without coronary artery lesions.
CAL, coronary artery lesions; CI, confidence interval; IVGG, intravenous gammaglobulin.

Table 3 Multivariate analysis of risk factors for coronary artery lesions

Risk factor (cut-off point for categorical variable)	Exclusion of white blood cell count post-additional IVGG (n = 44)		Inclusion of white blood cell count post-additional IVGG (n = 39)	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Febrile days pre-additional IVGG (<10 days/≥10 days)	7.86 (1.44 to 42.8)	0.02	25.6 (2.31 to 289.1)	0.008
Febrile days post-additional IVGG (<2 days/≥2 days)	4.42 (0.82 to 23.8)	0.08	5.90 (0.90 to 38.9)	0.07
Number of days that initial IVGG was divided over (1 or 2 days/4 or 5 days)	3.40 (0.44 to 26.3)	0.24	3.16 (0.44 to 29.2)	0.31
White blood cell count pre-additional IVGG (<16 000/μl/≥16 000/μl)	4.19 (0.79 to 22.2)	0.09	3.00 (0.38 to 23.9)	0.30
C reactive protein pre-additional IVGG (<9.7 mg/dl/≥9.7 mg/dl)	1.71 (0.20 to 14.7)	0.63	1.79 (0.15 to 21.1)	0.64
White blood cell count post-additional IVGG (<11 300/μl/≥11 300/μl)	–	–	9.35 (0.93 to 94.3)	0.06

CI, confidence interval; IVGG, intravenous gammaglobulin.

risk factors for CAL in initial IVGG non-responders, such as age <6 months, male sex, high white blood cell count, and high C reactive protein concentration in the present study.

In order to start the infusion of additional IVGG before the first 10 febrile days, we make three recommendations to physicians who treat patients with acute KD. First, KD patients should be treated immediately with an initial IVGG infusion. Although the efficacy of early treatment before five days of illness is controversial,[20] Tse and colleagues[21] reported that treatment with initial IVGG on or before the fifth illness day resulted in better coronary outcome. Second, initial IVGG should be given in a single infusion, because 4–5 daily infusions led to delayed administration of additional IVGG. In Japan, the medical insurance system previously prevented physicians from treating with a single infusion despite the proven efficacy,[9] but it has recently allowed its use. Third, it should be judged early whether patients respond to initial IVGG or not. However, we agree with Burns and colleagues[1] and Brogan and colleagues[7] that patients with fever at least 48 hours after completion of a single infusion of initial IVGG are considered non-responders, since occasional responders have fever during the period. If additional IVGG is given 48 hours after completion of the infusion before the first 10 febrile days, initial IVGG should be completed by the first seven febrile days.

The present study has some limitations. There may be unknown selection bias owing to the retrospective nature of the study. A prospective randomised control study would be optimal to establish the efficacy of additional IVGG for patients who do not respond to initial IVGG, but it would be difficult to justify inclusion of an untreated control group in view of the increased risk of CAL in patients with persistent or recrudescent fever. We used four or five daily infusions of IVGG, which is not currently recommended in other countries.[7–9] The administration of various brands of IVGG may be a confounding factor.[20]

In our study, among KD patients with persistent or recrudescent fever after initial IVGG therapy, administration of additional IVGG before the first 10 febrile days was associated with a decreased risk for CAL, when compared with the risk in those who were retreated later. This finding provides support for early diagnosis and initial treatment of KD and suggests that an additional IVGG infusion, if administered early, may prevent CAL in initial IVGG non-responders.

ACKNOWLEDGEMENTS

We are grateful to Dr Anne H Rowley of the Departments of Pediatrics and Microbiology/Immunology at Northwestern University Feinberg School of Medicine, and Dr Mitio Inokuti of Argonne National Laboratory for critical reading of an earlier manuscript. We are indebted to Dr Hiroshi Yanagawa of Saitama Prefectural University and Dr Yosikazu Nakamura of the Department of Public Health at Jichi Medical School for providing data of nationwide epidemiologic surveys on the incidence of Kawasaki disease in Japan. Furthermore, we would like to thank T Takahashi, Y Kojima, M Satoh, T Murai, M Ihara, J Ishihara, M Tukumura, H Toyoma, T Furuta, H Fukushima, J Maeda, E Takahashi, H Ueda, and S Yoshiba at Keio University School of Medicine for their excellent cooperation. The following institutions participated in the present study: Keio University School of Medicine, Tokyo; Tokyo Metropolitan Kiyose Children's Hospital, Tokyo; Tokyo Metropolitan Ohtsuka Hospital, Tokyo; Kawasaki Municipal Hospital, Kawasaki; Yokohama Municipal Citizens' Hospital, Yokohama; Yokohama Rosai Hospital, Yokohama; Saitama Municipal Hospital, Saitama; Ischara Kyodo Hospital, Ischara; Hiratsuka City Hospital, Hiratsuka; Hiratsuka Kyosai Hospital, Hiratsuka; Ashikaga Red Cross Hospital, Ashikaga; Saiseikai Utunomiya Hospital, Utunomiya.

Authors' affiliations
M Miura, H Ohki, T Tsuchihashi, H Yamagishi, Y Katada, K Yamada, Y Yamashita, A Sugaya, O Komiyama, H Shiro, Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

REFERENCES

1 **Burns JC**, Capparelli EV, Brown JA, for the US/Canadian Kawasaki Syndrome Study Group, *et al.* Intravenous gamma-globulin treatment and retreatment in Kawasaki disease. *Pediatr Infect Dis J* 1998;17:1144–8.
2 **Han RK**, Silverman ED, Newman A, *et al.* Management and outcome of persistent or recurrent fever after initial intravenous gamma globulin therapy in acute Kawasaki disease. *Arch Pediatr Adolesc Med* 2000;154:694–9.
3 **Durongpisitkul K**, Soongswang J, Laohaprasitiporn D, *et al.* Immunoglobulin failure and retreatment in Kawasaki disease. *Pediatr Cardiol* 2003;24:145–8.
4 **Sundel RP**, Burns JC, Baker A, *et al.* Gamma globulin re-treatment in Kawasaki disease. *J Pediatr* 1993;123:657–9.
5 **Hashino K**, Ishii M, Iemura M, *et al.* Re-treatment for immune globulin-resistant Kawasaki disease: a comparative study of additional immune globulin and steroid pulse therapy. *Pediatr Int* 2001;43:211–17.
6 **Wallace CA**, French JW, Kahn SJ, *et al.* Initial intravenous gammaglobulin treatment failure in Kawasaki disease. *Pediatrics* 2000;105:E78.
7 **Brogan P**, Bose A, Burgner D, *et al.* Kawasaki disease: an evidence based approach to diagnosis, treatment, and proposals for future research. *Arch Dis Child* 2002;86:286–90.
8 **Rowley AH**, Shulman ST. Kawasaki syndrome. *Pediatr Clin North Am* 1999;46:313–29.
9 **Newburger JW**, Takahashi M, Beiser AS, *et al.* A single intravenous infusion of gamma globulin as compared with four infusions in the treatment of acute Kawasaki syndrome. *N Engl J Med* 1991;324:1633–9.
10 **Beiser AS**, Takahashi M, Baker AL, *et al.* For the US multicenter Kawasaki disease study group. A predictive instrument for coronary artery aneurysms in Kawasaki disease. *Am J Cardiol* 1998;81:1116–20.
11 **Koren G**, Lavi S, Rose V, *et al.* Kawasaki disease: review of risk factors for coronary aneurysms. *J Pediatr* 1986;108:388–92.
12 **Zaitsu M**, Hamasaki Y, Toshiro K, *et al.* Ulinastatin, an elastase inhibitor, inhibits the increases mRNA expression of prostaglandin H2 synthase-type 2 in Kawasaki disease. *J Infect Dis* 2000;181:1101–9.

13 **De Zorzi A**, Colan SD, Gauvreau K, *et al*. Coronary artery dimensions may be misclassified as normal in Kawasaki disease. *J Pediatr* 1998;133:254–8.
14 **Rosenfeld EA**, Corydon KE, Shulman ST. Kawasaki disease in infants less than one year of age. *J Pediatr* 1995;126:524–9.
15 **Ayusawa M**, Harada K. Appropriate use of γ-globulin for Kawasaki disease [in Japanese]. *Shoniko* 1995;36:461–6.
16 **Naoe S**, Takahashi K, Masuda H, *et al*. Kawasaki disease with particular emphasis on arterial lesions. *Acta Pathol Jpn* 1991;41:785–97.
17 **Kato H**. Cardiovascular involvement in Kawasaki disease: evaluation and natural story. In: Shulman ST, ed. *Kawasaki disease*. New York: Alan R, Liss, 1987:277–86.
18 **Mori M**, Imagawa T, Yasui K, *et al*. Predictors of coronary artery lesions after intravenous γ-globulin treatment in Kawasaki disease. *J Pediatr* 2000;137:177–80.
19 **Miura M**, Kajima Y, Satoh M, *et al*. Development of criteria to determine risk of coronary artery aneurysms in patients with Kawasaki disease after intravenous gamma globulin treatment [in Japanese]. *Pediatr Cardiol Cardiac Surg* 1997;13:12–18.
20 **Newburger JW**, Taubert KA, Shulman ST, *et al*. Summary and abstracts of the seventh international Kawasaki disease symposium. *Pediatr Res* 2003;53:153–7.
21 **Tse SM**, Silverman ED, McCrindle BW, *et al*. Early treatment with intravenous immunoglobulin in patients with Kawasaki disease. *J Pediatr* 2002;140:450–5.