H-Y ANTIGEN STUDIES IN THIRTY PATIENTS WITH ABNORMAL GONADAL DIFFERENTIATION: CORRELATIONS AMONG SEX CHROMOSOME COMPLEMENT, H-Y ANTIGEN, AND GONADAL TYPE

Kiyomi YAMADA[1] and Koichiro ISURUGI[2]

[1]*Division of Genetics, Clinical Research Institute, and*
[2]*Department of Urology, National Medical Center Hospital,*
1-Toyamacho, Shinjuku-ku, Tokyo 162, Japan

Summary   The expression of H-Y antigen was examined in 30 patients with abnormal gonadal differentiation.   Four patients with XX true hermaphroditism and 8 with XX male syndrome were all H-Y positive, in spite of the absence of cells bearing Y chromosome in the body.   Of the 7 patients studied with XY gonadal dysgenesis, 6 patients were H-Y positive and one was negative.   The reduced antigen titer as compared to normal males was observed in one of the H-Y positive patients.   Clinical and cytogenetic studies in the total 30 patients adding 4 of mixed gonadal dysgenesis and 3 of male pseudohermaphroditism, could not establish strict correlations among sex chromosome complement, H-Y antigen, and gonadal type of patients.   However, a distinct association was recognized; when testicular structure was seen in the gonad, the H-Y antigen of the patient was always positive regardless of sex chromosome complement. In this study, our modified method and technical problems on the H-Y antigen examination are described, and genetic mechanisms for the regulation of H-Y antigen expression are discussed.

INTRODUCTION

Various kinds of abnormal sex chromosome complement had been revealed in man, and consequently, an important role of the Y chromosome in differentiation of the embryonal gonad was well acknowledged.   Namely, it was generally admitted that the Y chromosome is a strong testis determinant and in its absence an ovary is formed.   However, XX males and XX true hermaphrodites among human sex anomalies were found to be the only exception to this assumption, both having an XX sex complement and testicular structure in the gonad.   To explain these disease conditions, mosaicism or Y-X translocation had been considered as most likely (Fergusson-Smith, 1966).

A male-specific antigen was discovered by Eichwald and Silmser (1955) in the mouse, which was later called as histocompatibility-Y (H-Y) antigen. The first application of H-Y antigen examination to humans was reported by Wachtel et al. (1976) and revealed that XX males and XX true hermaphrodites were H-Y antigen positive. The H-Y antigen is a cell surface component and is thought to be controlled by a Y-chromosomal gene. In parallel with accumulation of experimental evidence supporting that H-Y antigen may be the testis-determining factor in mammals, rapid expansion of knowledge on H-Y antigen phenotype in various human sex-anomaly patients was made and the underlying genetic mechanisms now come to the time to be discussed actively (see the review of Wachtel and Ohno, 1979).

Methodological problems on the examination of H-Y antigen, however, are still existent for many reasons. In the present study, we wish to describe our modified method for the H-Y antigen detection in detail and the results of H-Y antigen studies in 30 sex-anomaly patients including XX males and XX true hermaphrodites. Technical problems, clinical and cytogenetic correlations, and genetic mechanisms of the H-Y antigen expression are discussed.

MATERIALS AND METHODS

The chromosome examination was done in cells obtained by the conventional blood-culture method. Peripheral leukocytes were also used for H-Y antigen typing. The examination of H-Y antigen was performed according to the sperm cytotoxicity test of Goldberg et al. (1971) with following modifications by us.

1) Production of H-Y antiserum in mice. An inbred strain of C57BL/6 mice was used. Each of female virgin mice aged 7–9 weeks was injected weekly with 1 ml of 0.85% saline containing 3–5×10^7 male spleen cells intraperitoneally. After 6 serial injections, blood was taken 7 days after the last injection. The serum was heat-inactivated, and stored at $-70°C$ until just before use.

2) Preparation of antiserum for use. Eight ml of heparinized blood of the patient being tested or normal control persons was mixed with 2 ml of 5% dextran in saline, and allowed to stand for 30 min in a test tube. The upper part of the blood containing leukocytes richly was centrifuged at 1,000 rpm for 10 min. The cells were rinsed twice with 0.02% EDTA-Hanks' solution. Absorption of the H-Y antibody was accomplished by suspending over 10^7 leukocytes in 100 μl of antiserum diluted 1/4 with saline in a small test tube and by allowing the suspension on ice for 50 min. After centrifuged at 500 g for 10 min, the supernatant serum was used in cytotoxicity tests.

3) Preparation for sperm suspension. The epididymes were removed from an adult BALB/c mouse, sliced into several pieces, and suspended in 0.5 ml of RPMI 1640 medium containing 5% of heat-inactivated fetal calf serum. The upper part of the suspension containing free sperm was taken, diluted to a concentration of

5×10^6/ml, and immediately used in cytotoxicity tests.

4) Preparation of rabbit serum. Selected rabbit serum was used as complement source. One ml of serum diluted 1/4 with saline containing 0.01 M EDTA was mixed with liver and spleen cells of a female BALB/c mouse in an approximate proportion of four volume serum to one volume packed cells. The cell suspension in a small tube was placed on ice for 50 min to absorb the rabbit anti-mouse antibody occurring naturally. After centrifuged at 500 g for 10 min, the absorbed serum was further diluted 1/2 or 1/3 with saline containing 0.02 M Ca- and Mg-ions.

5) Sperm cytotoxicity test. Antiserum aliquots after absorption with leukocytes of the patient being tested or of normal human males and females as controls, were tested for their residual cytotoxicity against mouse sperm. Each volume (20 µl) of i) antiserum (serially diluted 1/4 to 1/32), ii) sperm suspension, and iii) rabbit serum, were mixed in a small test tube and incubated at 37°C for 50 min. Besides duplicated sets of antiserum absorbed with patient's cells, the following four series with antiserum treated differently were set up as controls; absorbed with human normal male or female leukocytes, unabsorbed, and no antiserum. Sperm counts were made 10 min after addition of 20 µl of a freshly prepared solution of 0.2% trypan-blue in saline. Sperm stained with the dye were scored as dead under observation with a Normarski interference phase contrast microscope (Olympus Inc.). Usually, 100 sperm per test tube were quickly counted.

RESULTS AND DISCUSSION

1. H-Y antigen studies in normal controls

In Fig. 1, normal ranges of human male and female curves in cytotoxicity tests are graphically shown. From the results of normal controls, it was clear that at 1/4 and 1/8 dilutions of antiserum the residual cytotoxic activities after absorption with XY cells were significantly lower than those after absorption with XX cells.

2. H-Y antigen studies in 30 sex-anomaly patients

Results of H-Y antigen typing and chromosome analysis, together with information on the gonads of patients studied, are summarized in Table 1. Data in cytotoxicity tests of patients with XX true hermaphroditism, XX male syndrome, and XY gonadal dysgenesis are graphically shown in Fig. 2, respectively.

XX true hermaphroditism and XX male syndrome. We studied 4 cases of XX true hermaphroditism and 8 of XX male syndrome. All of them (Cases 1–12) were H-Y antigen positive. Indeed, there was no exception among over 20 previously reported cases of these diseases (Wachtel *et al.*, 1976a; Fraccaro *et al.*, 1979). Y-X translocation or mosaicism with cells bearing Y chromosome was not observed in the present cases, though the abnormal X (Xp+) chromosome suggesting Y-X translocation was reported only in a few cases (Evans *et al.*, 1979). Therefore, it is natural to consider that gene(s) responsible for the expression of H-Y antigen
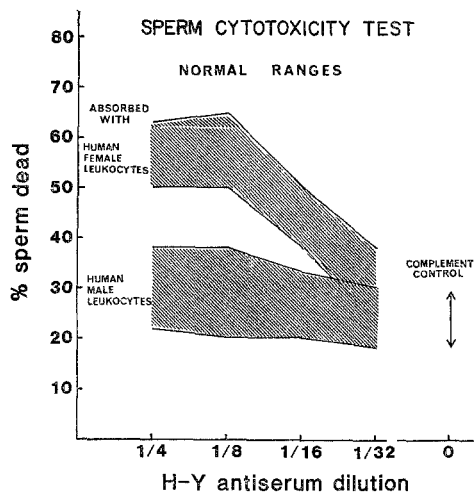
Fig. 1. The proportion of dead sperm according to serial dilutions
of antiserum; a normal range for females (obtained from
the total 20 tests from 6 individuals), and a normal range
for males (obtained from the total 20 tests from 8 indi-
viduals).

should exist within a 46,XX genome of these patients.   The presence of reported
cases in which XX true hermaphroditism and XX male syndrome were both found
in the same family, has suggested a common genetic basis for the two disease condi-
tions and an autosomal mode of inheritance in the families reported (Berger *et al.*,
1970; Kasdan *et al.*, 1973).

XY gonadal dysgenesis.   We studied 7 cases of XY gonadal dysgenesis (Cases
13-19).   Of the 7 cases studied, 6 cases were H-Y positive and one (Case 17) was
negative.   The reduced antigen titer was observed in one (Case 13) of the positive
cases.   Our present results were similar to the results of previously reported cases;
of the 24 cases reported, 16 positive, 6 negative, and 2 were positive with the reduced
antigen titer (Ghosh *et al.*, 1978; Wolf, 1979; Wachtel *et al.*, 1980a).

*Analysis of correlations among sex chromosome complement, H-Y antigen, and
gonadal type.*   Data summarized in Table 1, led us to the conclusion that there was
no correlation in the strict sense among karyotype, H-Y antigen, and gonadal type
of the 30 patients studied here.   However, it was noted that when testicular develop-
ment was seen in the gonad, the H-Y antigen was always positive regardless of sex
chromosome complement.   In other words, none of patients was observed, having
a testis without the expression of H-Y antigen on peripheral leukocytes.   This
distinct association is also valid in the light of evidence obtained in reported cases.

3.   Genetic mechanisms for the regulation of H-Y antigen expression
Since the gene for human H-Y antigen is throught to be located on the short
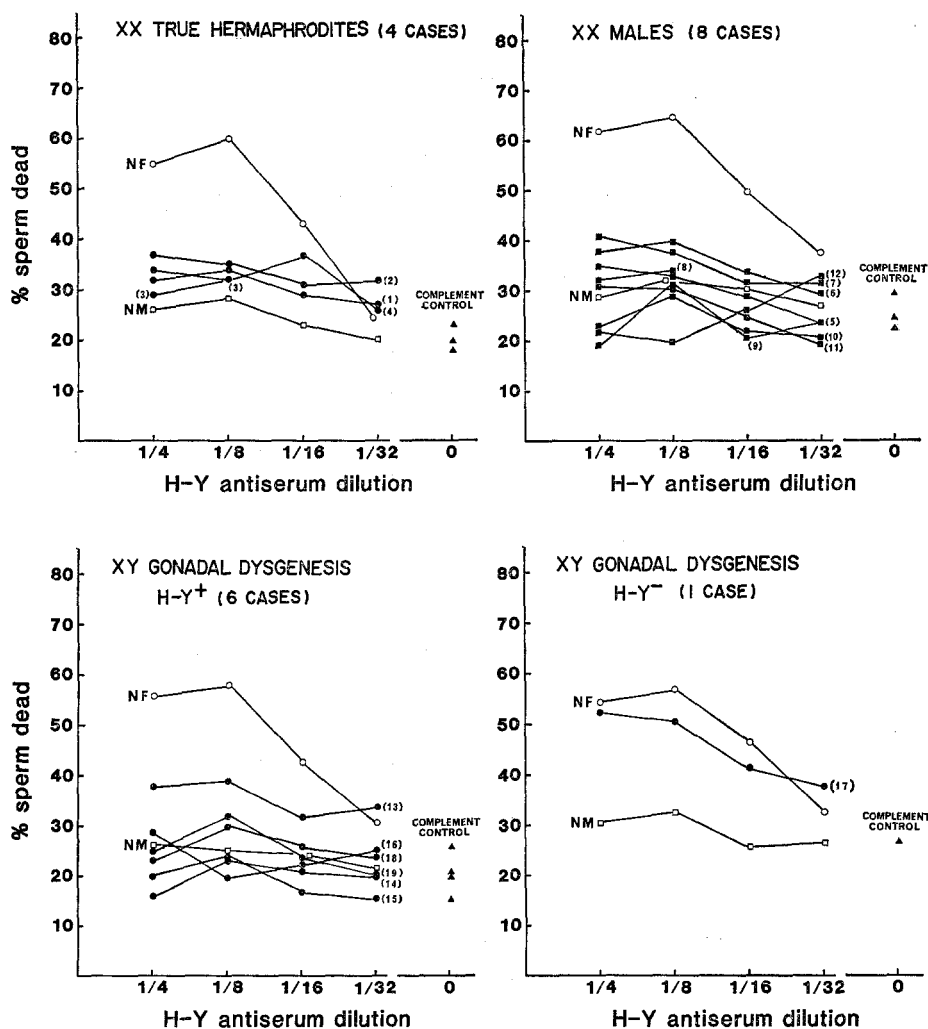
Fig. 2. Determination of H-Y phenotype by residual cytotoxic activities of antiserum absorbed with
patient's leukocytes.   Numbers in parentheses are Case Nos., and curves of normal female
(NF) and normal male(NM) are expressed as a mean of total controls.

arm of the Y chromosome (Koo *et al.*, 1977), present results of Cases 21–23 and
26 supporting this, coincidence between the presence of Y chromosome and the
presence of H-Y antigen or testis in the gonad is well explained.   When this coin-
cidence is disturbed by mutation, various forms of intersexuality come out.   Accord-
ingly, information on genetic mechanisms in the H-Y antigen system is obtained
from studies in patients with abnormal gonadal differentiation.

Observations to date in intersexuality of mammals including man, which can
not be explained by such an idea as only the Y chromosome having the gene for the

Table 1. Results of analyses on H-Y antigen, karyotype, and gonadal type in 30 patients studied here

Case No.	Age	Phenotypic Sex	Clinical Diagnosis	H-Y Antigen	Karyotype	Type of the Gonads
1	10y	Male	True hermaphroditism	H-Y$^+$	46,XX	{ Rt:Ovotestis Lt:Ovotestis
2	21y	Male	True hermaphroditism	H-Y$^+$	46,XX	{ Rt:Ovotestis Lt:Ovotestis
3	1y	Male	True hermaphroditism	H-Y$^+$	46,XX	{ Rt:? Lt:?
4	2y	Male	True hermaphroditism	H-Y$^+$	46,XX	{ Rt:Ovotestis Lt:Ovotestis
5	7y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
6	4y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
7	9y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
8	40y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
9	29y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
10	28y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
11	17y	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
12	6m	Male	XX male syndrome	H-Y$^+$	46,XX	{ Rt:Testis Lt:Testis
13	3y	Female	XY gonadal dysgenesis	H-Y$^\pm$	46,XY	{ Rt:Gonadoblastoma Lt:Dysgerminoma
14	28y	Female	XY gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Streak Lt:Streak
15	26y	Female	XY gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Streak Lt:Streak
16	8y	Female	XY gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Gonadoblastoma Lt:Gonadoblastoma
17	3y	Female	XY gonadal dysgenesis	H-Y$^-$	46,XY	{ Rt:? Lt:?
18	17y	Female	XY gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Streak Lt:Streak
19	17y	Female	XY gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Streak Lt:Streak
20	3y	Female	Mixed gonadal dysgenesis	H-Y$^+$	46,XY	{ Rt:Testis Lt:Streak
21	45y	Female	Mixed gonadal dysgenesis	H-Y$^+$	45,X/46,XYq-	{ Rt:Streak Lt:Testis
22	1y	Ambiguous	Mixed gonadal dysgenesis ?	H-Y$^+$	45,X/46,X idic(Yq)	{ Rt:Absence Lt:Testis
23	2m	Ambiguous	Mixed gonadal dysgenesis ?	H-Y$^+$	45,X/46,XYq-	{ Rt:Testis Lt:Aggregates of germ cells ?
24	40y	Female	Male pseudohermaphroditism	H-Y$^+$	46,XY	{ Rt:Testis Lt:Testis(seminoma)
25	2y	Ambiguous	Male pseudohermaphroditism	H-Y$^+$	46,XY	{ Rt:Testis Lt:Testis
26	21y	Female	Male pseudohermaphroditism ?	H-Y$^+$	45,X/46,X tan dup(Yq)	{ Rt:Testis ? Lt:Testis ?
27	22y	Male	Hypogonadism	H-Y$^+$	46,XYq-	{ Rt:Testis Lt:Testis
28	35y	Male	Hypogonadism	H-Y$^+$	46,XYq-	{ Rt:Testis Lt:Testis
29	10y	Ambiguous	Klinefelter syndrome	H-Y$^+$	49,XXXXY	{ Rt:Testis Lt:Testis
30	65y	Male	Leukemia	H-Y$^+$	46,Xinv(Yq)*	{ Rt:Testis Lt:Testis

Cases 5-6, and 14-15, are sibs. Cases 18-19 are monozygotic twins. *The chromosome anomaly was constitutional.

regulation of the H-Y expression, may be summarized briefly as follows: i) Human XX true hermaphrodites and XX males are invariably H-Y positive. The analogy to this, is seen in dogs (Selden *et al.*, 1978), mice (Bennett *et al.*, 1977), and goats (Wachtel *et al.*, 1978). ii) Though the majority of human patients with XY gonadal dysgenesis are H-Y positive, the minority are H-Y negative. Fertile XY females, being H-Y negative, are reported in the wood lemming (Wachtel *et al.*, 1976b). iii) Human patients with Turner syndrome having 45,X or 46,Xi(Xq) or 46,XXp− karyotype are invariably H-Y positive, the antigen titer being reduced as compared to normal males (Wolf *et al.*, 1980; Wachtel *et al.*, 1980b). The same condition to this, is reported in 39,X mice (Engle *et al.*, 1981). iv) Inducible effects of H-Y antigen to testicular differentiation depend on a threshold.

The most acceptable postulate, at present, which is able to explain all of the above findings without discrepancy, is a genetic hypothesis which has been proposed by Wolf (Wolf, 1980; Wolf *et al.*, 1980): The expression of an autosomally localized *structural* gene for H-Y antigen is regulated by an X-linked *repressor* gene and by a Y-linked *inducer* gene. According to the Wolf's hypothesis, XX true hermaphroditism and XX male syndrome may be due to mutation of the X-linked repressor gene, and H-Y negative cases of XY gonadal dysgenesis may be caused by mutation of the Y-linked inducer gene. H-Y positive cases of XY gonadal dysgenesis can be explained as a H-Y antigen receptor defect. Under this hypothesis, in normal XX females the repressor gene should not undergo inactivation and its gene locus is tentatively assigned to Xp223 from analysis of an X/Y translocation case (Wolf *et al.*, 1980). The evidence that genes on the Xp223 escape X-inactivation, has been obtained by other studies (Tiepolo *et al.*, 1980). As for the locus of the H-Y structural gene, of particular interest are the recent reports that three XY females with campomelic dysplasia, a disease inherited as an autosomal recessive trait, were found to be H-Y negative (Bricarelli *et al.*, 1981; Puck *et al.*, 1981).

4. Technical problems on the H-Y antigen examination

Though several methods for the H-Y antigen detection already have been reported, common technical problems seem to exist in any method; complicated technical procedures and low reproducibility of the results obtained. These are mostly due to the reason that H-Y antiserum is low titered and contaminated with autoantibodies. In the present study, it was fairly easy to determine the H-Y phenotype as positive or negative, but comparison of antigen titers between different patients was impossible due to fluctuation of control baselines. Since the quantitative difference of H-Y antigen titers has recently become an important problem, the reliable method of quantitative assays is now needed. So that, the recent report of high titers of monoclonal antibody obtained by hybridoma technique (Koo *et al.*, 1981), has brought us an expectation to develop more easy, reliable methods such as radioimmunoassays or direct immunofluorescence method in the very near future.

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