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H-Y Antigen and Sex Determination [and Discussion]

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H-Y antigen and sex determination

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The primary development of a male rather than a female gonad in mammals is determined by the presence of a Y chromosome. The other property unique to the Y chromosome is the occurrence of a cell-surface antigen (designated H-Y) which distinguishes male from female. Thus it was determined that male grafts were rejected by otherwise histocompatible females of the same inbred strain and later that H-Y-specific cytolytic T cells were produced by these grafted mice. When it was determined that females grafted with male skin produced antibody defining a serologically detectable male antigen (which may or may not be the same as H-Y), further immunogenetic analysis of this antigenic system became possible in terms of humoral and cellular factors. By using this assay it was demonstrated that the antigen was phylogenetically conserved and that it was expressed in the male mouse embryo as early as the 8-cell stage of development. The notion that H-Y was a single molecular species responsible for triggering the indifferent gonad to differentiate into the testis became a widely accepted hypothesis. In this report the H-Y antigenic system is traced historically from its original description to the role played in testis development. Data are presented which suggest that although H-Y is a male-specific factor and may play a role in male sex determination, it is unlikely that it is the primary inducer of testis differentiation.

1. History of H-Y antigen

The H-Y antigen was originally defined by the ability of female mice to reject otherwise histocompatible male skin of the same inbred strain (Eichwald & Silmser 1955). In fact, the antigen was discovered by serendipity because the original design of Eichwald & Silmser's experiment was to look at the phenomenon of tolerance. C57BL/6(B6) mice were selected because of the inability to induce acquired immunological tolerance in this strain via the intra-uterine route of antigen administration. Without exception B6 male skin grafted to B6 female mice was rejected. Because the incompatibility between male and female was thought to be somehow linked to a gene or genes on the Y chromosome, the antigen responsible was designated H-Y (Billingham & Silvers 1960).

In the case of H-Y, in contrast to other alloantigenic systems, there was no conclusive serological demonstration of the antigen. Because this limited further genetic analysis and also restricted additional studies regarding the nature of the H-Y immune response in terms of cellular and humoral factors, we decided to determine whether or not the antigen could be detected serologically. Because of our interest in sperm-surface antigens and also because H-Y was male specific, the complement-mediated cytotoxic test was applied to mouse spermatozoa for the detection of a male-specific antigen, possibly the same as H-Y. Serum raised in B6 female mice that had rejected several male grafts was used with rabbit serum as a source of complement for the detection of male-specific antigen on B6 sperm. Results from this early work clearly showed that male-specific antigen was detectable serologically by the cytotoxicity

test (hereafter denoted sperm cytotoxicity assay) *in vitro* (Goldberg *et al.* 1971), and secondly that it was present on sperm as was suggested earlier by indirect evidence (Katsh *et al.* 1964). This assay opened the floodgates to further analysis of this male-specific antigen. By using the sperm cytotoxicity assay it was demonstrated that the antigen was phylogenetically conserved, being expressed in cells from XY males of the guinea pig, human, rabbit and rat (Wachtel *et al.* 1975*a*; Wachtel 1983). For this reason it was proposed that H-Y antigen (whether the transplantation H-Y or the serologically detected male antigen was not discussed) played a critical role in male sex differentiation (Wachtel *et al.* 1975*b*; Ohno & Matsunaga 1981). This hypothesis was supported by the finding that serologically detected male antigen was expressed in the male embryo as early as the 8-cell stage of development in the mouse. By using the complement-dependent cytotoxicity assay and male-specific antiserum it was shown that 50% of 8-cell mouse embryos were lysed (Krco & Goldberg 1976). Affected embryos were male as demonstrated by chromosomal analysis in which more than 90% of the unaffected embryos were XX (Epstein *et al.* 1980), and also by embryo transfer techniques in which 82% of offspring resulting from transfer of unaffected embryos to surrogate mothers were female (Shelton & Goldberg 1984).

To provide an *in vitro* parallel to the rejection of male skin grafts, we modified the cell-mediated cytotoxicity (CMC) assay for the lysis of male target cells by H-Y specific cytolytic T lymphocytes (CTL) (Goldberg *et al.* 1973). Spleen cells from B6 female mice that were grafted with male skin served as a source of H-Y specific CTLs which were reacted with ^{51}Cr-labelled male lymph-node target cells; the results clearly indicated a specific lysis of male cells. This assay was further modified and extended by Gordon *et al.* (1975) to show that susceptibility of the male target cell to lysis was restricted by genes within the Major Histocompatibility Complex (Simpson & Gordon 1977; von Boehmer & Haas 1979). H-Y-specific T-cell clones were subsequently produced from immunized female mice (von Boehmer *et al.* 1979; Tomonari 1983; Simpson *et al.* 1984) and more recently human H-Y specific CTL clones have been described (Goulmy 1985). By using CTL clones in proliferation assays and cell-mediated cytotoxicity tests, it has been possible to H-Y type sex-reversed mice (McLaren *et al.* 1984) and humans (Simpson *et al.* 1987). The cell-mediated cytotoxicity assay has proved useful for providing an *in vitro* correlate to skin-graft rejection and has therefore permitted further analysis of H-Y, especially as it relates to sex determination.

(*a*) *Does H-Y represent a single molecular species?*

The male-specific antigen identified by serological assays and the H-Y antigen originally described by female rejection of male skin were thought to be the same (i.e. H-Y was a single molecular species). This assumption was questioned by Melvold *et al.* (1977) based on the finding of a single XO mutant male mouse. The mouse was H-Y negative by transplantation experiments and skin from this mouse was accepted by 13 females of the same strain. Moreover it rejected male skin much like females of that strain. On the other hand, it was positive for male-specific antigen when typed serologically. On the basis of this finding, it was concluded that two distinct male-specific antigenic systems existed, one recognized by transplantation assays and the other detectable serologically. In addition, it provided evidence that the structural gene for the serologically detected male-specific antigen was not on the Y chromosome.

Further support that there were two distinct male-specific antigens resulted from findings that XO female mice expressed the serologically detectable antigen (Koo *et al.* 1983; Engel

et al. 1981) despite the fact that these females were able to reject male skin (Simpson *et al.* 1982). In addition, H-Y-specific CTLs were unable to lyse cells donated by XO mice, indicating the absence of H-Y (Simpson *et al.* 1982). These studies supported the concept that two distinct male-specific antigenic systems existed, one detectable by transplantation and cell-mediated immunological assays (designated H-Y) and the second recognized serologically (designated the serologically detectable male antigen (SDMA) (Silvers *et al.* 1982)). However, more recently a conflicting report appeared in which SDMA-negative XO female mice were described, suggesting again that H-Y and SDMA are the same (Wiberg & Mayerova 1985). Thus the question of multiplicity of the H-Y antigenic system is not yet resolved.

With the use of molecular probes and biochemical technology, the question of whether or not H-Y represents one or more antigens should be answered in the near future. Results recently reported by Lau *et al.* (1987) suggest that a gene encoding the serologically detected male antigen, designated 'male enhanced antigen (MEA)' gene, has been isolated from a mouse complementary DNA (cDNA) library. Interestingly, it was shown that the gene for MEA was conserved among the genome of several mammalian species including guinea pig, rabbit, bull, dog and human. However, additional technical controls and experiments designed to determine specificity need to be done before it can be concluded that this gene encodes SDMA.

It has been proposed recently that the abbreviation 'Sxs' (serological sex specific antigen) (Wiberg 1987) be used when referring to SDMA because the serological antigen is detectable in a variety of species in which the heterogametic sex is female. However, for this report, SDMA is used throughout to define the male-specific antigen detected by antibody-mediated reactions. (It should be noted that the above discussion does not preclude the possibility that there are more than two male-specific antigens.)

2. H-Y AND SDM ANTIGENS: ARE THEY INVOLVED IN TESTICULAR DIFFERENTIATION?

The presence of a Y chromosome is almost always associated with testicular differentiation in mammals: hence XY, XXY and XYY individuals develop as males, whereas XX and XO individuals develop as females. As pointed out by Eicher & Washburn (1986), there are clearly non-Y-chromosome genes involved in testicular differentiation, but the initial event is most likely encoded by a Y-linked gene designated testis-determining-Y (or *Tdy*). Using Y-chromosome-specific DNA probes, Page *et al.* (1987) recently found that human males with the incongruous XX karyotype bear a small part of the short arm of the Y chromosome and that females with the XY karyotype lack that part of the Y, indicating that this portion includes a gene or genes encoding the testis determining factor (TDF). A central question addressed here is whether the gene encoding TDF is the same as that which encodes the SDM or H-Y antigens. Evidence is given here in mouse and man that neither antigen is TDF, although indirect evidence may implicate these antigens in later events of testicular development.

(*a*) *Evidence supporting the hypothesis that H-Y or* SDMA *or both identify the testicular determining factor*

Phylogenetic conservation of the antigen and expression of SDMA on the early male mouse embryo (§1) has been used as indirect evidence that SDMA is involved in testis development (for additional reviews see Nakamura *et al.* (1987); Polani & Adinolfi (1983); Zenzes & Reed

(1984)). Direct evidence supporting the concept that SDMA served as a signal for testicular development came from experiments in which it was demonstrated that disaggregated newborn rodent testicular cells reassociated to form structures that were morphologically and histologically similar to seminiferous tubules, and that H-Y-specific antibody blocked the development of these structures (Ohno *et al.* 1978). Moreover, *in vitro* treatment of disaggregated newborn rat ovarian tissue with soluble SDMA induced the formation of testicular structures (Ohno *et al.* 1979; Muller & Urban 1981).

Sex-reversed mice that carry the sex-determining locus of the Y chromosome, designated Sxr, on the distal end of the paternal X chromosome have been described (Evans *et al.* 1982; Singh & Jones 1982). Although these mice are of the XX genotype, they are indisputably male. They typed positive for the H-Y and SDMAs by cell-mediated cytotoxicity and transplantation assays as well as by sperm cytotoxicity tests (Bennett *et al.* 1977; Simpson *et al.* 1981). These findings supported the proposition that H-Y or SDMA or both play a role in testis induction, perhaps as a product of the testis-determining Y gene.

However, reports of work using sex-reversed mice and humans demonstrated, in several situations, a lack of correlation between H-Y antigen expression and phenotypic sex, thereby challenging the original proposal that H-Y, but not SDMA, was the trigger for male testicular differentiation.

(*b*) *Evidence against H-Y being the testicular determining factor*

Eicher developed a strain of mouse that provided one of the earlier clues that H-Y as detected by cell-mediated assays and transplantation techniques was not the product of *Tdy*. She and colleagues (Eicher *et al.* 1982) were able to transfer the Y chromosome from *Mus poschiavinus* to the C57BL/6 background (denoted C57BL/6Y^{POS}), causing XY individuals to develop either as females with two ovaries or as hermaphrodites. None of the XY individuals developed normal testes. What is relevant to the argument presented here, is that C57BL/6-Y^{POS} XY females were H-Y positive as determined by transplantation assays (i.e. XY^{POS} female skin was rejected by XX female recipients as rapidly as skin donated by normal XY males; Silvers *et al.* 1986), indicating that H-Y was not acting as a signal for testicular differentiation. It was suggested that inappropriate interactions between the *M. poschiavinus* Y-linked *Tdy* gene and other non-Y-linked testis-determining genes on the B6 genomic background resulted in abnormal testis differentiation; thus the H-Y antigen might remain intact but be non-functional (Ohno 1985).

XXSxr mice in which the Sxr-bearing chromosome is partnered by an X-autosome translocated chromosome, abbreviated T16H/X^{Sxr}, can develop as males, females or hermaphrodites (McLaren & Monk 1982; Cattanach *et al.* 1982). Presumably this is due to preferential inactivation of the Sxr-bearing X chromosome in which there is variable inactivation of the Sxr segment. By using T-cell clones in proliferation assays and H-Y specific CTL-mediated reactions *in vitro*, T16H/X^{Sxr} females were shown to be H-Y antigen positive. However, one variant female derived from this stock was H-Y negative (Simpson *et al.* 1986; Wiberg & Lattermann 1987) and when mated to a normal male gave rise to XXSxr sons that lacked H-Y (McLaren *et al.* 1984). The authors conclude that this variant form of Sxr (designated Sxr′) retained the testis-inducing signal while losing the gene or genes encoding H-Y. In fact, these XXSxr′ males are able to reject male skin (Simpson *et al.* 1986), further supporting the finding that H-Y antigen is not expressed despite testicular differentiation in this experimental situation. Interestingly, Burgoyne *et al.* (1986) showed that in contrast to

XOSxr males, the testes of XOSxr′ males lack spermatocytes and spermatids, indicating an almost total block of spermatogenesis. These authors suggest that the segment of Sxr either mutated or lost in the Sxr′ DNA may contain the gene or genes responsible for spermatogenesis and that this gene may be the same as, or closely linked to, that encoding H-Y.

The finding that the genes encoding H-Y antigen and TDF were separable was confirmed in humans by Simpson *et al.* (1987), using human H-Y-specific CTL clones and sex-reversed individuals. Six XX males who carried part of the distal short arm of the Y chromosome necessary for testicular differentiation as described above (Page *et al.* 1987) were H-Y negative, whereas two XY females lacking this portion of the Y short arm were indisputably H-Y positive.

Although the findings described here indicate that H-Y, at least as detected by skin-graft rejection and cell-mediated assays, is not the primary sex determinant in mouse and humans, a central question is whether SDMA is the factor responsible for testicular development.

(*c*) *Evidence against SDMA being the testicular determining factor*

To determine the SDMA phenotype of XXSxr′ males, three coded groups of XX female mice were grafted respectively with skin from XXSxr males, XXSxr′ males or XX females; their serum was then tested for the presence of sperm cytotoxic antibody. The results summarized in table 1 show that a significant number of females immunized with XXSxr, but not with

Table 1. Detection of antibody produced against SDMA by using serum from B6 female mice immunized with tissue from XX females, XXSxr males or XXSxr′ males

(Absorption of serum with male and female tissue demonstrated specificity of the reaction for SDMA. Clearly then, neither H-Y nor SDM antigens identify a determinant involved in testicular differentiation (summarized in table 2), but may well be involved in later events of testis development.)

B6 female mice immunized with tissue from[a]	presence of SDMA antibody[b]
XX females	−
XXSxr males	+
XXSxr′ males	−

[a] All mice were initially inoculated with spleen cells from either XX females, XXSxr males or XXSxr′ males, followed by a skin graft from a donor of the same genotype 13 days later. All mice received a subsequent intraperitoneal injection of donor spleen cells 14–25 days before serum collection.

[b] As determined by sperm cytotoxicity assay against BALB/c epididymal sperm.

Table 2. Summary of H-Y and SDM antigen expression in mice of various karyotypes and phenotypic sex

karyotype	phenotypic sex	H-Y *phenotype*[a]	SDMA *phenotype*
XX	female	−	−
XY	male	+	+
XXSxr	male	+	+
XXSxr′	male	−	−
XY^{POS}	female	+	not determined
XO	female	−	±[b]

[a] +, Presence; −, absence.

[b] XO females have been typed SDMA-positive and SDMA-negative in different studies (see §1*a*).

XXSxr′, male tissue produced antibody that was cytotoxic to sperm (Ellen Goldberg, Anne McLaren and Brian Reilly, unpublished results). H-Y and SDMA phenotypes in mice of various phenotypes and karyotypic sex are shown in table 2.

Conclusion

The original description of H-Y by skin grafting experiments and the serological identification of H-Y opened an exciting and important field of study related to male sexual development. Controversies currently exist because of lack of identification of molecular factors involved in the events associated with testicular development. Now, with the application of molecular probes and biochemical technology, together with the availability of sex-reversed mice and humans, much of the controversy will be resolved and the role of H-Y in sexual differentiation will be determined.

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Discussion

M. Adinolfi (*Paediatric Research Unit, Guy's Hospital, London, U.K.*). I understand that Dr Goldberg has not been able to detect H-Y antigen on fresh sperms by immunofluorescence; however, she has detected H-Y molecules by immunofluorescent technique on mouse blastocyst at 8- to 15-cell stage. Does she think that the H-Y antigen is synthesized by these cells and, if so, what can be the biological function of these molecules at such an early stage of development?

Ellen H. Goldberg. H-Y antigen was detected serologically on the 8-cell mouse embryo by the complement-mediated cytotoxicity assay (Krco & Goldberg 1976). We did not use immunofluorescent labelling techniques for the detection of the antigen. When the 4-cell embryo was examined for the presence of H-Y it was not detectable. For this reason, we feel that the antigen is synthesized by the embryo and is not an adsorbed product of the sperm tail. In addition, further work demonstrated that H-Y, as detected serologically, is expressed by the male embryo.

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U. Wiberg (*Institut für Humangenetik, Freiburg, F.R.G.*). Referring to Dr Goldberg's transplantation experiments with Sxr and Sxr′ males, to try to raise anti-Sxr antisera did she absorb such antisera with testis of Sxr and Sxr′?

Ellen H. Goldberg. To test for specificity for SDMA, antisera raised in B6 females against tissue from Sxr and Sxr′ males were absorbed with B6 male and female spleen cells before being tested for the presence of antibody to SDMA. The antisera were not absorbed with testes from Sxr and Sxr′ mice.

M. Bradley (*Medical School, University of Otago, New Zealand*). Firstly, does Dr Goldberg see killing of more than 50% of sperm in cytotoxic assay?

Has she tried using fluorescent-labelled antibodies to map distribution of H-Y on sperm?

Ellen H. Goldberg. Firstly, yes, we routinely see killing of more than 50% of sperm in the cytotoxic assay using H-Y specific antiserum and rabbit serum as a source of complement. However, there is variability from test to test and also among sperm from different mouse strains.

No, we have not tried using fluorescent-labelled antibodies to map the distribution of H-Y on sperm. However, Koo (1973), by using tobacco mosaic virus as a marker, was able to visualize a reaction on the acrosomal cap of mouse sperm by using immunoelectronmicroscopy and H-Y-specific antiserum.

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H. Sharma (71 *Barrack Road*, *Hounslow*, *U.K.*). Have cell lines been tested for H-Y positivity? Do any cancers produce 'aberrant' H-Y expression? Can Y-chromosomes or other chromosomes be introduced in H-Y negative cells to study H-Y expression?

Ellen H. Goldberg. Yes, cell lines have been tested for H-Y antigen expression and in some laboratories have been found to be positive. We have tested several murine tumour cell lines for the expression of sdma and have found variable expression of the antigen depending on the line tested. We cannot determine with the serological assay 'aberrant' expression of the antigen. We have no experience in determining whether or not cells in which the Y-chromosome was introduced will express the H-Y antigen. To study H-Y further in terms of gene expression, it would be useful to be able to transfect H-Y negative cell lines and look for the expression of H-Y.

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