IMMUNOGENETIC STUDIES ON H-Y ANTIGEN

by

Abraham Shalev

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

WINNIPEG , MANITOBA

February 1980

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A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
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To my son, Amichai

Acknowledgement

This page would be too short if I was to thank individually all those who in one way or the other furnished their moral or material support.

The people to whom I am particularly indebt are: Dr. J. L. Hamerton, my supervisor Dr. I. Berczi, co-supervisor and the one who provided me with laboratory space, Drs. J. S. Winter, S. S. Wachtel, and N. A. Nelson who offered their professional advice, Clara Shalev, my wife who never saved her time to offer whatever help needed and the members of my advisory committee.

A special word of thanks is due to the Faculty members and staff from the departments of Genetics, Anatomy, Endocrinology, the Computer department for HSC, and Central Animal Care Unit,HSC. , all of whom provided valuable assistance on many occasions during my research.

The work presented in this Thesis was supported by Grants from the Medical Research Council (MT. 4061) to Dr. J. L. Hamerton; The Children's Hospital of Winnipeg Research Foundation (Dr. J. L. Hamerton); St. Boniface Hospital Research Foundation (Dr's I. Berczi and J. L. Hamerton) and a grant in-aid from the Sigma Xi Foundation (A. Shalev). Support was also received for special projects through grants to co-investigators.

Contribution Statement

I, the undersigned state that all the studies presented in this thesis are based on my original ideas , my own work and my own writing. The following contributions were made by others in the joint papers:

Chapter II — Mr. P.Z. Goldenberg prepared the lobster hemocytes and contributed to the writing of the manuscript. Dr.E.Huebner identified and isolated the insects gonadal tissues and contributed to the writing of the manuscript.

Chapter III — Dr. E.Huebner identified and isolated the cells from the guppy and also contributed to the writing of the manuscript.

Chapter IV — Dr's. F.I.Reyes and J.Blankstein performed the surgery and provided the tissue for study. Dr. J.S.D. Winter and Dr. C.Faiman contributed in discussions and assisted in the writing of the manuscript. The hormonal studies were carried out by all the named above.

Chapter V — Dr.R.V. Short provided the goat cells and made comments on the early version of the manuscript. Dr.J.L.Hamerton was responsible for general

planning and co-ordination of the study. A substantial assistance in the writing of the manuscript was also provided by Dr. J.L.Hamerton.

Chapter VI Dr. C.K.Hannan was responsible for all the tests of HBsAg's which were carried out in this study. The planning of the experimental design and the writing of the manuscript were equally shared between Dr. Hannan and myself.

Dr. N.A.Nelson provided statistical advice on several occasions.

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THESIS CONTENTS

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ABSTRACT

1. A single restimulation of female DA and Lewis rats at 6 weeks after 10 consecutive injections with male splenocytes resulted in production of specific H-Y antisera. Complete Freund's adjuvant (CFA) was used prior to immunization of C57Bl/6 mice to induce high volumes of ascites fluid. The titer of H-Y antibodies in the ascites fluid was similar to the titer in the blood serum from a second group immunized without FCA.

2. The presence of a mammalian crossreactive H-Y antigen in the male lobster (Homarus americanus) is demonstrated by repeated testing of hemocytes from male and female lobsters. In addition evidence is presented for the presence of H-Y antigen on the surface of gonadal cells in three insect species. It is suggested that the mammalian H-Y antigen evolved from a primordial H-Y-like molecule in

invertebrates.

3. H-Y antigen has been detected in the guppy (Lebistes reticulatus) . The possible significance of H-Y antigen in fish is discussed in relation to its role in mammalian sex determination.

4. Cultured skin fibroblasts and gonadal cells (gonadoblastoma) from a patient with 46,XY gonadal dysgenesis were found to be H-Y positive. It is suggested that a mutation at a different locus (gonadal receptor?) could be responsible for this condition.

5. A family of polled goats was tested for H-Y antigen. Cultured fibroblasts from two homozygous (P/P) pseudohermaphrodite goats were found to express H-Y antigen at a lower level than fibroblasts from two other males. One heterozygous female (mother of an intersex) was found to express H-Y antigen at a subnormal level. Another heterozygous female was H-Y antigen negative. The possible genetic relation between the gene for polledness, the intersexuality trait and H-Y antigen are discussed.

6. No evidence was found for crossreactivity between the mosaic of Hepatitis B surface antigens and H-Y antigen as postulated by Drew et al. (1978, 1978a) Alternative possibilities to explain their observations of changes in sex ratio in the highly infected Greek population are suggested.

7. Splenectomy prior breeding of C57Bl/6 female mice did not alter their capacity to reject male skin grafts. However, it did result in a decrease in sex ratio.

8. Male skin graft survival in primiparous C57Bl/6 mice was

significantly shorter in females transplanted within 6 days post-partum as compared to females transplanted within 10 to 20 days post-partum. It is suggested that male fetuses stimulate the mother against H-Y antigen and that the immune balance between cytotoxic and tolerogenic (suppressor) cells changes with time after birth.

9. Parous DA rats produce H-Y antibodies in response to the stimulation of their male fetuses. Within the limitations of the size of the population studied, it was found that presence of antibodies was not correlated to the stage of parity or number of male offspring born. Maternal H-Y antibodies were transmitted to the fetuses and were detected only in female offspring. The possible significance of these findings is discussed.

10. Experiments were conducted to detect the possible effect of immunization to the H-Y antigen on sex ratio in C57Bl/6 mice. It was found that immunization to H-Y antigen following splenectomy resulted in a slight ,but significant, increase in sex ratio which was in good agreement with previous reports. Immunization to H-Y antigen without previous splenectomy or following sensitization with female cells did not affect sex ratio. Restimulation to H-Y antigen 30 days after the primary immunization resulted in a decrease in sex ratio. Other factors, such as litter size, breeding efficiency, and breeding+gestation period were not affected by any of the treatments. It is concluded that the maternal immune system plays a limited role in balancing the natural sex ratio in mice and that H-Y antigen may be of special significance in this respect.

PART 1

GENERAL INTRODUCTION

1.1 H-Y ANTIGEN:HISTORICAL ASPECTS

The histocompatibility Y (H-Y) antigen was discovered by Eichwald and Silmser (1955) who observed that male-to-female skin grafts within inbred strains of mice were rejected. From 1955 to 1972 male-to-female skin transplantation was the chief method employed to investigate H-Y antigen. Although the applications of this methodology were limited, some substantial understanding on the nature of H-Y antigen was achieved during those years which then allowed further progress applying *in vitro* methods. The achievements during this period have been discussed by Gasser and Silvers (1972), Silvers and Wachtel (1977), and Wachtel(1977) and are listed below .

1. A male specific (H-Y) antigen was detected on every tissue examined including skin,lung,salivary gland,blood leukocytes,red blood cells,spleen,liver,kidney,heart,parathyroid,thyroid, adrenal cortical tissue,pituitary glands, embryonic cells (11 days), and spermatozoa. H-Y antigen was therefore considered to have a ubiquitous distribution.

2.Expression of H-Y antigen in the mouse depends on the presence of a Y chromosome in the cells since XO cells or tumor cells lacking the Y fail to induce accelerated rejection of male skin grafts.

3.Expression of H-Y antigen is evidently unrelated to sex hormones.

4.Although all strains of mice react immunologically to H-Y antigen,some strains do not reject male skin grafts. The capacity to reject male skin grafts was associated with the murine major histocompatibility complex (MHC). It was concluded that genes in this region and probably also in other regions control the cytotoxic

response to H-Y antigen.

5. Experimental evidence suggested that Y-linked incompatibility between male fetuses and their mother has a stimulatory effect on placental growth and can affect sex ratio.

6. Tolerance to H-Y antigen can easily be induced in a variety of ways. Tolerance due to parity was particularly investigated.

7. A system homologous to the murine H-Y occurs in rats, chickens, and fish.

8. Existence of an X-linked histocompatibility antigen (H-X) in mice was studied.

The development of the sperm cytotoxicity test for H-Y typing (Goldberg et al., 1971) and the application of this test by Wachtel and his associates led to a series of discoveries which established the basis for most of the present research in this field. In an early study Wachtel et al. (1973) showed that male lymphoid cells resident in female (chimeric) mice continued to express H-Y antigen. This was definite evidence that expression of H-Y antigen is independent of male hormones. Shortly afterwards Wachtel et al. (1974,1975a) established the crossreactivity of H-Y antigen among a variety of vertebrate species from man to amphibia. Expression of H-Y antigen was found to be associated with the heterogametic sex irrespective of whether this was male or female . This finding and other evidence led Wachtel et al. (1975b) to propose for the first time that H-Y antigen is responsible for primary sex determination in mammals. A strict association between H-Y antigen and the presence of testicular tissue was confirmed in studies on 'accidents' of Nature in sex determination such as the XX males. Tfm mice and humans, the Sxr mouse, the freemartin and others (see Wachtel and Ohno (1979), Wachtel et al.

1977b ,and Ohno,1979).

Dr. Susumu Ohno (Ohno 1976a,1976b,1977a,1977b ,1979, Ohno et al. 1977, 1979) was the pioneer in proposing the physiochemical role of H-Y antigen in sex determination and its association with other plasma membrane components before firm experimental evidence was available. His models and hypotheses have been largely confirmed by later experiments.

Two biological phenomena which have accumulated a significant amount of evidence are: 1. the genetic control of cell mediated cytotoxic responses to H-Y antigen and 2. the association of H-Y antigen with other membrane components on the surface of the cells. In a series of publications ,Gordon et al. 1976,1977, Simpson et al. ,1978, Hurme et al. 1977, 1978a,1978b, Matsunaga and Simpson, 1978 and Goulmy et al. 1977) it was demonstrated that the cytotoxic response to H-Y antigen is restricted by compatibility at the MHC region in both mouse and man. Furthermore, several regulatory genes have been identified: an Ir gene at the MHC and other genes which were not linked to the MHC. These studies reveal that like other responses to histocompatibility antigens, the immune response to H-Y antigen is under genetic control, and furthermore, that H-Y antigenicity might be the result of its association with other membrane components such as β_2-microglobulin and MHC antigens . Several authors have investigated the association of H-Y with MHC antigens and 2 microglobulin on the plasma membrane. Geib et al. (1976) and Fellous et al. (1978) suggested that H-Y antigen is not associated with either H-2 or HLA antigens on the cell membrane, Beutler et al. (1972) suggested that H-Y antigen utilizes β_2-microglobulin-HLA antigen dimers as the plasma

membrane anchorage sites on human male cells (Ohno 1977). The association between H-Y and β_2-microglobulin has been substantiated also by Fellous et al (1978).

1.2 IN VITRO DETECTION OF H-Y ANTIGEN

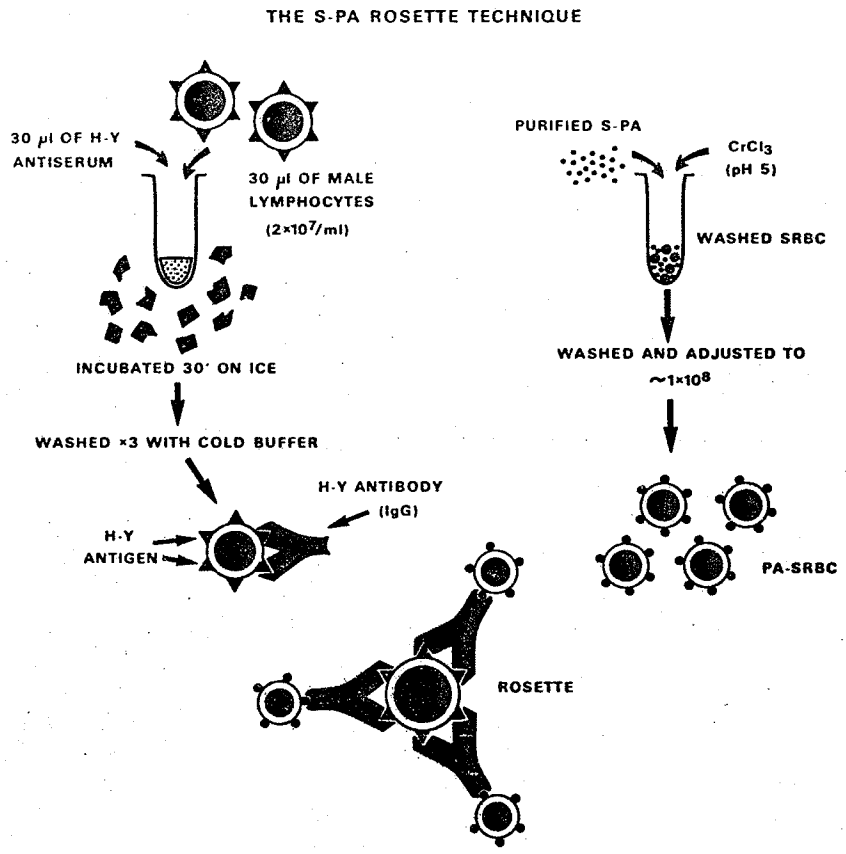
A serious difficulty encountered in the in vitro detection of H-Y antigen is the fact that H-Y is a weak antigen and results in low-titer antisera upon immunization. The general problem of "weak" vs. "strong" antigenicity, although interesting, is not pertinent to the subject of this thesis. However, attention is directed to a recent publication in which the immunogenicity of antigens is shown to be in opposite relation to their evolutionary conservation rate (Ogievetskaya, 1979). A general advantage for studies on H-Y antigen is that inbred strains of probably any species and cells from most tissues may be used for immunization. A great variety of in vitro detection methods have been suggested in recent years (see Shalev et al. 1978). However, none of the methods was sensitive enough for introduction into routine clinical diagnosis and therefore remained within the limits of laboratory experimentation. The technical diffculty of using and testing H-Y antisera is demonstrated by the fact that H-Y antisera are rapidly inactivated at room temperature or after repeated freeze-thawing . This sensitivity restricts the way in which antisera can be used in various immunological techniques, . Until the development of more sensitive methods such as radioimmunoassay, one is left with rather 'crude' qualitative detection methods which are of limited use in quantitation studies.

The fact that H-Y antisera are able to kill spermatozoa (complement mediated cytotoxicity)led Shalev et al. (1977) to develop a method which allowed observation of the fluorescent X (Barr) and Y (or F) bodies in live cells following the selective killing of some cells by complement and antibody. This technique enables one to test the specificity with which cells expressing Y-linked (H-Y) antigens or X-linked antigen are killed in heterogeneous cell population such as sperm,tumor cells or cultured cells. Anti H-Y serum was not applicable in this study since H-Y antibodies are not known to be cytotoxic to fibroblasts and staining of sperm for Y-bodies was least succesful. A direct hemagglutination technique for H-Y typing was also developed by Shalev et al. (1978),but has not been used in studies presented in this thesis because of inherent technical difficulties(i.e. time consuming) and because of the advantage of the protein - A rosette technique for detection of maternally transmitted (IgG)antibodies. The PA rosette technique which was modified of the technique of Koo and Goldberg (1978) has been used in all in vitro studies presented in this thesis. The principles of the method are illustrated in Figure 1 and the modifications from Koo and Goldberg (1978) are listed below.

1.Preparation of 0.1% $CrCl_3$ stock solution: A glass container,glass stopper and a stirrer, were soaked for 48 hours in chromic acid to remove debris and then washed for several hours under running distilled water. Hand contact was avoided. Two gms. of $CrCl_3$ crystals are disolved in 200 ml. of saline (prepared from triple distilled water) stirred for several hours. This solution was adjusted to pH 5.0 daily for at least one week with a NaOH 0.01N solution. Further adjustments were made every second week. The solution should be green-blue in color and completely clear. Prior to conjugation of

Figure 1.

The principles of the S-PA rosette technique.



Stages in the technique

1. Conjugation of staphylococcal protein A (S-PA) to washed sheep erythrocytes (SRBC) and checking for successful conjugation (absence of clumps and high rosette formation with a known antiserum).

2. Incubation of male and female lymphocytes in H-Y antiserum.

3. Washing of excess antiserum.

4. Adding of PA-SRBC to washed lymphocytes, spinning and incubation at room temperature.

5. The quality of the H-Y antiserum is determined by the efficiency of rosette formation with male as compared to female lymphocytes.

PA to the sheep erythrocytes (Koo and Goldberg,1978)the solution is diluted ten-fold in saline and used immediately.

2.Lymphocytes (from the same species and strain as the anti- H-Y sera) were routinely obtained from mesenteric and peripheral lymph nodes through maceration in minimal essential medium (MEM) or Hank's medium and transfered through a lightly packed nylon wool syringe to eliminate clumps of cells. Viability of at least 80% was confirmed prior to use by dye exclusion (trypan blue). Following the incubation of the lymphocytes with the antiserum they were washed twice with a volume of 50 ml. of ice cold (0-4°C)phosphate buffered saline (PBS) and then spun together with 0.3 ml. of 0.5% PA-SRBC (800 r.p.m.) for 10 min. (ratio of lymphocytes to SRBC ranged between 1:20 to 1:40). The pellet was allowed to stay at room temperature for 60 min. before final scoring for rosettes.

3. One drop (25 µl) of fresh crystal violet solution in PBS(2.0%) was added to the pellet which was then resuspended and allowed to stay at room temperature for another 2 min. Two hundred cells or more were regularly and randomly counted for each tube under a hemocytometer. Cells with at least 3 attached SRBC were counted as a possitive rosette.

These conditions allowed fair reproducibility of the results. Minimal variability was observed with repetitions of the same experimental tube (0-5%). The variability increased with the use of different batches of PA-SRBC and H-Y antisera, but mostly did not exceed 15%.

No convention exists for an optimal method(s) to produce H-Y

antisera, use of preferred species, or selection of a particular tissue to be used for immunization exists. However,it seems acceptable that doses of 0.5-3 x 10^6 cells are optimal for primary stimulation. Higher doses may lead to tolerance while lower doses might not produce a sufficiently strong response or may as well induce tolerance. The method of immunization should also be appropriate to the detection method used. For instance, a strong secondary response is optimal for use in stimulating production of IgG antibodies which is useful for the protein A rosette technique.

1.3 EVOLUTIONARY CONSERVATION OF H-Y ANTIGEN

One of the most interesting features of H-Y antigen is its extreme evolutionary conservation. H-Y is a cell membrane component that is wide spread among living organisms (vertebrates, invertebrates?,protozoa?) like the familiy of histone proteins (I,II 2,IIBI,III,IV), actin and cytochrome C,all of which show only slight amino acid changes in the course of millions of years of evolution (Ogieveskaya,1979).

Early transplantation experiments indicated that mouse and rat H-Y antigen are very similar or identical (Silvers and Young, 1973). Wachtel et al. (1975a) were the first to demonstrate crossreactivity of H-Y antigen in a great variety of vertebrate species including birds and amphibia. These findings were confirmed in a number of studies in which rat or mouse H-Y antisera were used to detect H-Y antigen in a variety of species (Shalev et al. 1978, Fellous et al. 1978). It is perhaps noteworthy that we had difficulties in

demonstrating presence of H-Y antigen on cells from the cat, the quail and the rainbow trout which may suggest major antigenic differences in these species (Shalev et al., 1978).

In other studies using the PA rosette technique, H-Y antigen was identified on cells derived from the male guppy (Lebistes reticulatus) and on hemocytes from the male lobster (Homarus americanus)(see below). Evidence for expression of a mammalian crossreactive H-Y antigen on the gonadal cells of three insect species is presented in Chapter 2.

What part of the H-Y molecule is identified as foreign by females which then mount an immune response? Ohno (1977a) suggested that the evolutionary conserved part of H-Y antigen (recognized by B cells)is different from the part of the antigen which is recognized by T cells and that both parts are embedded in an anchorage site composed of β_2-microglobulin and MHC antigen. As mentioned above the association between H-Y antigen and β_2-microglobulin was demonstrated but no good evidence for the association of H-Y with MHC antigen is equivocal. In light of the weak immune response to H-Y antigen, it can be speculated that some parts of the H-Y molecular complex may be recognized as self determinants. Indeed, evidence for the existence of a factor in fetal mouse ovary that can block binding of H-Y antigen molecules to ovarian cells has been recently presented (Wachtel and Hall, 1979).

Some experimental evidence suggests that the H-Y antigen on mouse skin (identified in vivo) may differ from the H-Y antigen on lymphoid cells which was recognized by in vitro techniques (Melvold, 1977, Haughton et al. 1979) and it is possible that there are

two different male specific antigens. Other questions of interest are whether or not the murine H-Y antigen has alleles (same species) and to what extent ,if at all, its structure is different in evolutionary distant classes such as Osteichthyes (bony fish) or Amphibia and Mammalia. Some claims for allelism of H-Y antigen have previously been made by Hildeman and his associates (see Wachtel,1977). However,in two recent studies (Andrews and Wachtel,1979; Simpson et al. 1979) attempts to discover allelism for H-Y antigen in outbred populations of mice failed. As far as molecular differences in the structure of H-Y antigen among various species or classes are concerned;no data are available.

1.4 H-Y ANTIGEN AND SEX DETERMINATION

The role of H-Y antigen in mammalian sex determination has been recently discussed and presented in a series of publications (Ohno,1977;Wachtel and Ohno (1979),Zenzes et al. 1978a,1978b) and Ohno et al. ,1979).

Most reviews on sex determination and the evolution of sex determination to date deal with sex chromosomes as physical and visual markers rather than with sex determining genes,their products and their mode of action.This approach is somewhat misleading, since the presence of sex chromosomes (particularly in invertebrates and low vertebrates) provides little,if any, information on the actual genetic and physiochemical control on sex determination. They may, however, be relevant to other functions such as spermatogenesis and ovogenesis (e.g. Drosophila). The absence of typical sex chromosomes,on the

other hand,does not necessarily indicate absence of a genetic control on sex determination which leads to the primary establishment of a sexually characterized gonadal tissue.The only direct approach to an understanding of the mechanism of sex determination is to identify the responsible gene products and to identify their role in this process. The discovery of the crucial involvement of H-Y antigen in mammalian sex determination is a major step in this direction.Does H-Y antigen have the same significant role in non-mammalian species as in mammals? In a recent study Muller et al. (1979) presented data showing that H-W(H-Y) antigen expression can be induced in gonads of sex-reversed male chicken embryos after oestradiol treatment.This finding suggests not only that the structural gene for H-Y(H-W) is not W-linked in the chicken, but also that there may be an interdependent interaction between the effect of H-Y antigen and the effect of the hormonal environment in determining the gonadal sex. Furthermore,this finding might suggest that the functional significance of H-Y antigen is related to the evolutionary stage of the class: H-Y may have little or no special significance for sex determination in lower vertebrates and invertebrates and may have acquired its major sex determining role in mammals, gradually.

1.5 FETAL-MATERNAL IMMUNE INTERACTION

Fetal-maternal immune interactions have been the subject of numerous investigations, papers and books. The central questions are how the conceptus ,as an allograft, survives the maternal immune reaction to its paternal derived antigens, and how does the immune

state of the mother influence its development.General reviews on this subject are available (Beer and Billingham ,1976, Hendenhall,1976, Billington, 1976, Gudson, 1976,Solomon, 1976, and Bernard, 1977).

In the preface to their book Beer and Billingham (1976) chronologically list the major events in the development of the research on the immunology of mammalian reproduction.It was only at the beginning of the 20th century that the fetus was first recognized as an allograft which in some mysterious way manages to thrive and escape the adversity of the maternal immune system.This question has not yet been completely resolved.It became clear that nature's defence mechanisms are not perfected and that under special circumstances damage and even destruction of the conceptus by the maternal immune system can occur. Furthermore,it became increasingly evident that histocompatibility differences between mother and conceptus can affect embryonic implantation,trophoblastic invasion, placental size and fetal weight. A milestone in the understanding of fetal-maternal interactions was the widely confirmed phenomenon of transfer of maternal and fetal cells through the placenta and the transmission of maternal antibodies to the fetus in most mammalian species including man. The consequences of maternal exposure to paternal antigen is well known in intercrosses of rat strains: neonates die due to runt disease (graft versus host) resulting from the maternal transmission of stimulated lymphocytes against paternal antigens,or as a result of maternal preconditioning to paternal strain skin grafts.Similar consequences are recognized also in humans (see Beer and Billingham,1976).

The general question of changes in sex ratio and other possible

roles of H-Y antigen in affecting the fetal-maternal immune interactions are discussed elsewhere in this thesis. The studies deal with the immune response to H-Y antigen of parous rats and mice as reflected by the production of H-Y antibodies , capacity to reject male skin grafts and changes in sex ratio.

PART 2

METHODOLOGY. EVOLUTIONARY CONSERVATION. SEX DETERMINATION.

CHAPTER I

A NOTE ON THE PRODUCTION OF H-Y ANTIBODIES

The most serious hindrance to the immune detection of H-Y antigen is the weakness of the antisera. This results in low cytotoxic titers to mouse sperm or low affinity to lymphocytes when measured by rosette formation. This characteristic of H-Y antisera limits its application in various immunological procedures such as antibody purification and cytotoxicity to lymphoid cells.

It is known that different species and strains of mice and rats can be used for production of H-Y antibodies, including those strains of mice which do not reject male skin grafts (Goldberg et al.: Hausman, 1973: Fellous et al., 1978)). The C57Bl/6 strain has been,however, most frequently used for this purpose (Wachtel et al., 1974; Wachtel et al., 1975; Wachtel et al. ,1976)). Nonetheless, the efficiency of various species, strains and immunization protocols to produce H-Y antibodies is almost an uninvestigated field. The present study is a modest contribution in this direction.

Using a modified technique for H-Y typing of Koo and Goldberg (1978)(see Introduction) the production of H-Y antibodies by inbred strains was compared after a series of injections (termed here 'primary response ') or a single re-stimulation (secondary response). In addition, a new immunization technique was examined in C57Bl/6 mice. One group was normally immunized and the other was given complete Freund's adjuvant (FCA) prior to immunization to generate ascites fluid.

Spleen cells for immunization were prepared as follows: Spleens from two mice or one rat were removed into 10 ml. of MEM medium. Using a fine needle and a 1 ml. syringe , the spleens were then perfused

with the same medium to force the erythrocytes into the medium. After 10 such prefusion injections the spleens were free of most (~80%) of the erythorcytes and were mechanically macerated. Before adjusting the concentration of cells they were passed through a non-packed nylon wool Pasteur-pipette to eliminate cell-clumps.

Female mice were given 5 intraperitoneal (i.p.) injections of $2-3x10^6$ male splenocytes in 0.1 ml. saline at 3 days intervals. A month later the animals were re-stimulated with 3 injections of the same concentration at 7 day intervals. The group treated with FCA was given 0.2 ml. of FCA i.p. 30 min. prior to the injections of cells which resulted in the development of an ascites in the second week of immunization. These animals were "milked" weekly by aspiration with 5 ml. syringe. The volume of ascites fluid obtained from each mouse during the three weeks was between 3 and 6 ml.

Female rats were given 10 consecutive injection (i.p.)of $2-3 \times 10^6$ male splenocytes and were re-stimulated 6 weeks later with a single dose of $1x10^7$ male splenocytes.

.The results in Table 1 indicate that the antisera obtained following the secondary immunization in both DA and Lewis rats were of higher specificity in binding to male cells. Accordingly, the means $\pm$ S.D. of the difference in rosette formation with male and female lymphocytes of DA and Lewis rats are 4.1 ± 7.4 and 2.3 ± 3.2 respectively in the primary response and 12.2 ± 5.0 and 12.0 ± 2.0 respectively in the secondary response. The efficiency of C57Bl/6 mice in production of H-Y antibodies with or without FCA is compared in Table 2. Although these groups might be too small to evaluate the efficiency of FCA as a means to stimulate a stronger response to H-Y

Table 1: Primary and secondary responses of DA and Lewis rats to H-Y antigen*

Animal	Primary response		Secondary response	
#	DA	Lewis	DA	Lewis
1	13.6(11.8)	6.1(4.2)	15.0(13.3)	12.3(7.9)
2	-6.1(25.7)	5.9(8.0)	19.3(10.1)	12.9(8.8)
3	10.0(6.8)	4.9(5.6)	12.6(12.2)	13.2(3.8)
4	-1.5(14.0)	0.2(4.6)	7.8(13.1)	11.3(7.4)
5	10.5(10.8)	1.2(11.8)	16.6(11.4)	14.3(7.4)
6	3.1(15.4)	-1.4(15.8)	6.0(25.5)	12.1(6.8)
7	-1.2(9.5)	-0.5(9.3)	8.3(9.2)	7.7(8.2)
normal serum	1.1(3.4)	-1.3(1.5)	-1.8(4.8)	0.3(3.2)

* Results (single scoring) are expressed as a difference in % rosettes formation with male and female lymphocytes (% rosettes with female cells in brackets).

Blood sera were collected 7 days after the final immunization. The same animals were used for the primary and secondary responses. The means $\pm$S.D. of rosette formation with male vs. female lymphocytes respectively are : 17.5$\pm$5.7 and 13.4$\pm$6.1 (DA, primary response), 10.7$\pm$3.5 and 8.5$\pm$4.2 (Lewis, primary response), 25.8$\pm$5.0 and 13.5$\pm$5.5 (DA, secondary response) and 19.3$\pm$2.2 and 7.2$\pm$1.6 (Lewis, secondary response).

Table 2: A secondary response to H-Y antigen of C57Bl/6 females with and without CFA*

Animal #	Group 1 (blood serum)	Group 2 (ascites fluid)
1	17.9(3.4)	17.3(4.1)
2	33.1(11.3)	0.6(8.6)
3	12.2(10.1)	14.1(19.2)
4	6.2(17.3)	18.9(12.5)
5	25.4(9.0)	4.5(6.3)
6	1.9(17.5)	12.4(2.9)
7	8.4(15.8)	2.1(6.3)
normal serum	-0.3(2.1)	0.6(2.5)

* Results are expressed as a difference in % rosette formation with male and female lymphocytes (% rosettes with female lymphocytes in brackets).

The ascites fluid and blood serum were collected from two different groups of mice 7 days after the last immunization. The mean±S.D. of rosette formation with male and female lymphocytes respecitvely are: 27.1±9.0 and 21.1±5.2 (group 1) and 18.5±10.4 and 8.6±5.6 (group 2).

antigen, an advantage for using ascites fluid is that it is a way to obtain higher volumes of antibody-containing fluid (by repeated 'milking') then can be obtained from blood serum.

A variety of tissues can be used to immunize females against H-Y antigen, since H-Y antigen seems to be ubiquitously distributed in male tissues (Gasser and Silvers, 1972)). Indeed the immunization of C57Bl/6 mice and DA rats with testicular cells, sperm, spleen cells, blood cells , and male skin grafts all led to the production of H-Y antibodies (Shalev et al., 1978) . In these studies antisera were collected and pooled from experimental groups of 8 animals (identically immunized) and studied for presence of autoantibodies. In light of the present finding, it is suggested that these autoantibodies were contributed by some animals whereas other animals produced good anti H-Y sera. The reasons for formation of autoantibodies are not clear. However, mechanisms such as modification of 'self' antigens due to the in vitro handling or feasible co-recognition of a modified "self" antigen in association with the recognition of H-Y antigen can be considered. Another aspect of interest is the reasons for 'incomplete' rosette formation (not 100%) with H-Y antisera. One can attribute this to a low concentration of H-Y antibodies (and therefore lower statistical chance for binding to the cells) or to low affinity between the lymphocytic H-Y antigen and H-Y antibodies. A low concentration of H-Y antigen on the cell surface may be considered as well.

From this study it is concluded that individual bleeding and testing of antisera is most crucial. Furthermore, pooling of antisera

could be wasteful in the sense that it may require prior absorptions with female cells and loss of specificity. Finally, production of strong and specific H-Y antiserum may be realized with more ease once the antigen is purified . The use of the hybridoma technique (monoclonal antibodies) should prove useful for H-Y antigen studies.

CHAPTER II

EVIDENCE FOR AN H-Y CROSSREACTIVE ANTIGEN IN INVERTEBRATES *

* In press in Differentiation. Done as a joint study by A.Shalev, P.Z.Goldenberg ,from the Department of Immunology, University of Manitoba, Manitoba Institute of Cell Biology, and Dr. E.Huebner, from the Department of Zoology , University of Manitoba.

Introduction

Sex specific antigens are a common feature in eucaryotic and procaryotic species including bacteria, protozoa, plants, insects, and mammals (Clarke et al., 1977: Crandall, 1977: Fox, 1958: Fox et al., 1962; Monroy and Rosati, 1979: Wachtel et al., 1975). Within this panoramic range of sex-specific antigens, the evolutionarily conserved H-Y antigen seems to have had a strong selective advantage in evolution. It is a histocompatibility antigen which is present on virtually all cells of male mammals (Gasser and Silvers, 1972) functioning in mammalian sex determination as a hormone-like substance (Muller et al., 1978; Ohno, 1979). The crossreactivity of the serologically detectable H-Y antigen has been demonstrated in a wide range of vertebrate organisms (Wachtel et al., 1975: Ohno, 1979: Shalev et al., 1978: Selden and Wachtel, 1977) which makes it likely to be an ubiquitous antigen among vertebrates. In repeated experiments, we observed that hemocytes from the male but not the female lobster specifically absorbed H-Y antibodies. The phenomenon of sex specific absorption was also found in three insect species.

Methods

The modified version of the Protein A (PA) rosette technique, was previously described (Part 1 and Chapter V).

Anti H-Y serum was produced in female Lewis rats by 10 injections (i.p.) of 2×10^6 male splenocytes at 2-3 day intervals followed by a booster of 1×10^7 cells given 6 weeks later. In previous tests this

antiserum showed high specificity in binding to rat male lymphocytes. Rat (Wistar) anti SRBC serum was received as a gift from Dr. E.Nagy and had a titer of 1/125 by direct hemagglutination with SRBC. Rat γ-globulin was purchased from Miles Laboratories. Inc. Anti H-Y serum was used at a dilution of 1:4, anti SRBC at a 1:2 dilution and rat γ-globulin at a 1:10 dilution (1.8 mg/ml at O.D. 280)

Hemocytes from 5 different pairs of male and female lobsters were isolated by withdrawing 1 ml. of lobster hemolymph from the ventral hemal sinus into a syringe containing 9 ml. lobster hemolymph medium (Paterson and Stewart, 1974) supplemented with 0.1 M cysteine . The cells were spun down for 10 min. at 25 g and 4°C.

Gonadal cells from 2 to 10 insects (depending on species and size of gonads) were mechanically macerated into 10 ml. of Grace's Insect Medium (Gibco #194G). The sex of the insects was determined externally and verified by the gonadal morphology and by microscopic examination of the gonad content. Gonadal cells were washed once in the same medium and spun down at 150 g and 4°C for 5 min.

An equal volume (30 µl.) of packed lobster hemocytes (volumetric measurment) or insect gonadal cells were used to absorb 30 µl. of antiserum. All cell samples were coded prior to testing and the results interpreted prior to identifying the sex of the donor animals.

Results and Discussion

In each of 10 consecutive tests, using coded hemocytes from 5 different pairs of male and female lobsters, only male hemocytes specifically absorbed rat H-Y antibody. A highly significant ($P<0.001$)

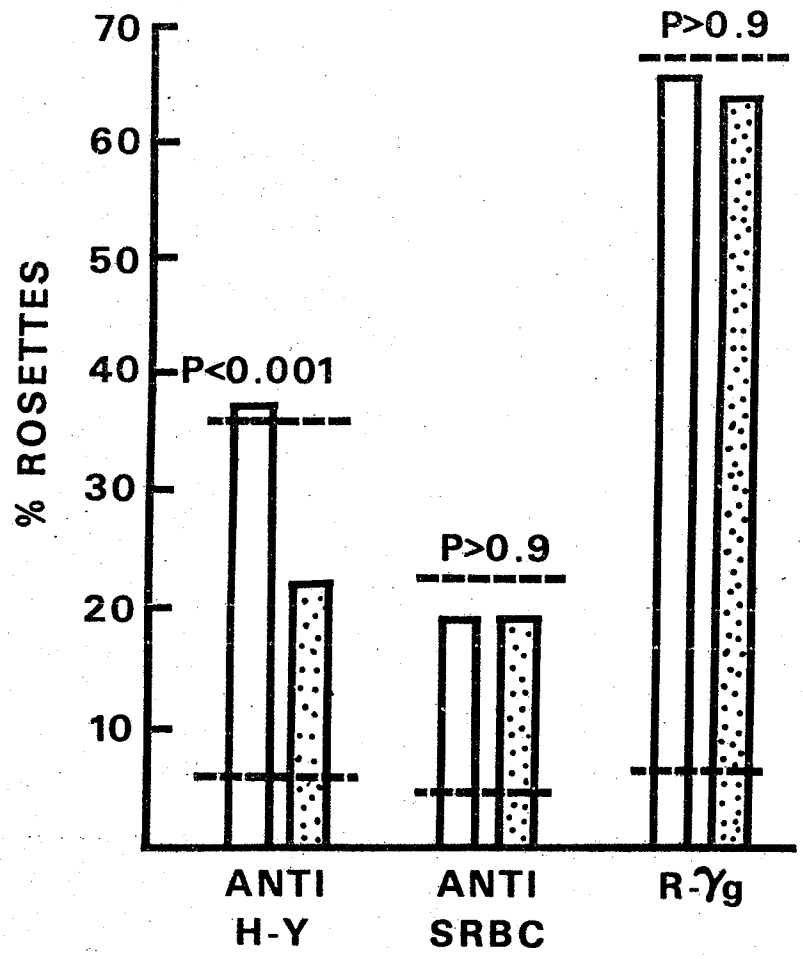
difference in rosette formation was observed between the male absorbed and female absorbed H-Y antiserum, but not with rat anti sheep red blood cells (SRBC) antiserum or rat γ-globulin (Figure 1). (Rosette formation with γG is probably due both to the presence of rat autoantibodies and to the binding of aggregated Ig to Fc receptors). To establish the quantitative nature of the absorption , lobster hemocytes and rat spleen cells were exponentially diluted and their capacity to absorb H-Y antibody was compared. As shown in Figure 2, reduction in rosette formation following the absorption with male hemocytes and male rat spleen cells was very similar, which strongly supports the hypothesis that the lobster's crossreactive antigen has a cellular distribution.

Absorption of rat H-Y antiserum with gonadal cells from three insect species indicates that the testicular cells from the Coleopteran beetle (Passalus cornutus) and the ovarian cells from two Orthopteran cockroaches (Leucophaea maderea and Diploptera punctata), but not gonadal cells from the opposite sex, absorbed H-Y antibodies to a significant extent (Figure 3). Therefore, an H-Y crossreactive antigen was present on the beetles testicular cells and the ovarian cells of the cockroaches.

In mammals, H-Y antigen is associated with the heterogametic sex (male). In species in which the female is heterogametic, an H-Y crossreactive antigen was found to be associated with the female (Wachtel et al., 1975: Ohno, 1979: Shalev et al., 1978). This association, although unexplained as yet, is of interest and might be related to the evolution of the genetic control on sex determination. The sex chromosomes of the lobster (H. americanus) are unknown,

Figure 1

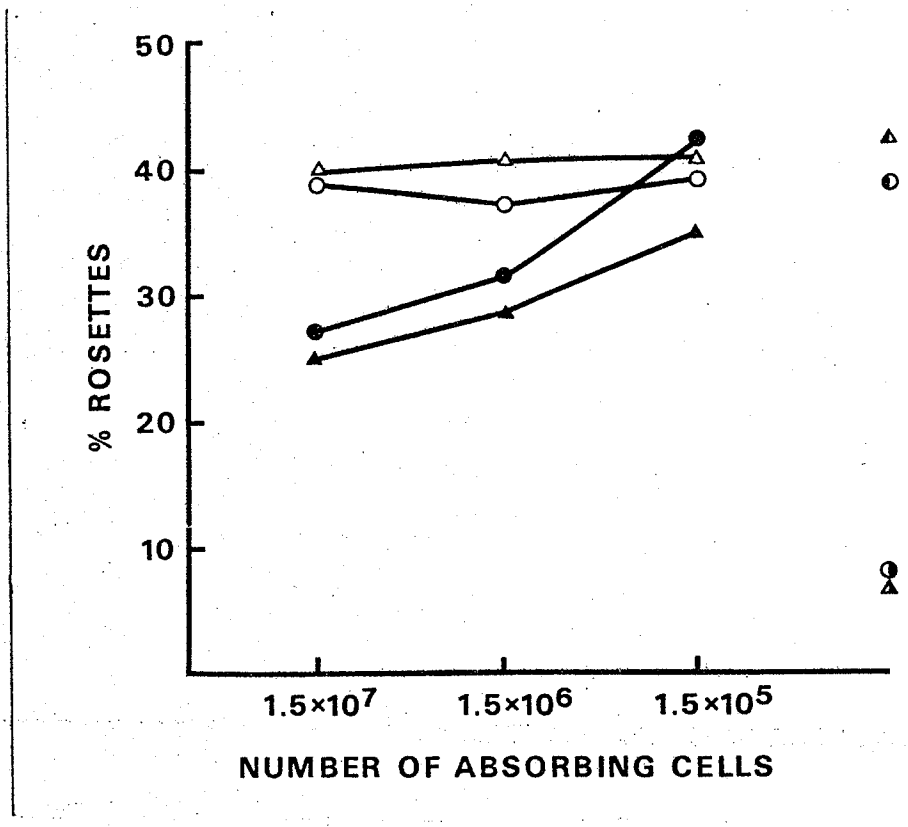
Presence of a male specific (H-Y) antigen on the surface of hemocytes from the male lobster.



Each bar represents the mean % rosette formation with male Lewis rat lymph-node lymphocytes following absorption of the antiserum with male (▨) or female (□) lobster hemocytes. Broken horizontal lines represent the mean of tests with unabsorbed antiserum (top) or no antiserum (bottom). The means±S.E. of male vs. female absorbed tests from 10 experiments with anti H-Y serum, 4 experiments with rat-anti-SRBC serum and 6 experiments with rat γ-globulin were compared by paired t-test and the results of the statistical analysis are indicated above. The mean±S.E. of the bars from left to right respectively are: 38.2±2.6, 21.3±2.4, 19.4±2.1, 19.3±0.9, 65.8±2.1 and 64.3±1.7. No self agglutination of the SRBC occured as a result of the use of anti-SRBC antiserum.

Figure 2

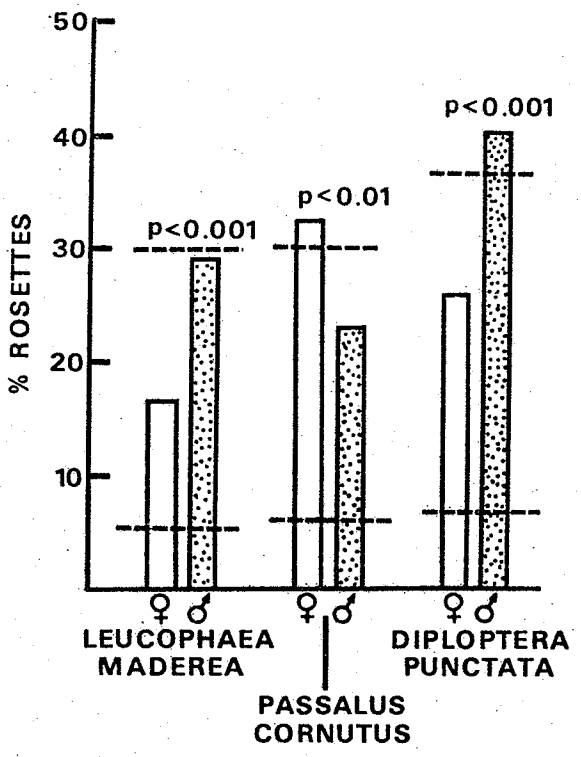
Quantitative absorption of anti H-Y serum with cells from male and female lobster and rat



The results represent the mean from two separate experiments. Spleen cells were obtained from male and female Lewis rats and were washed twice with 20 ml. of phosphate buffered saline prior to testing. Markings in the graph are as follows: absorbed male lobster hemocytes (●), absorbed female lobster hemocytes (○), absorbed male rat splenocytes (▲), absorbed female rat splenocytes (△), no absorption (◐) and antiserum omitted (◑), controls for lobster tests and no absorption (▲) and antiserum omitted (▲) controls for rats tests.

Figure 3

Absorption of rat anti H-Y sera with gonadal cells from three insects.



Each bar represents mean±S.E. of 11 tests with cells from Leucophaea maderia (L.M.), 6 tests with cells from Passalus cornutus (P.C.) and 8 tests with cells from Diploptera punctata (D.P.). The mean±S.E. of the male absorbed (▨) and female absorbed (□) tests respectively are: 16.7±2.4, 28.9±3.1 for L.M., 31.8±3.9, 22.4±4.3 for P.C., and 26.3±2.8, 41.0±3.7 for D.P. Broken horizontal lines represent the mean of controls with unabsorbed antiserum (top) and no antiserum (bottom). The results of statistical analysis (paired t-test) for each species are indicated.

however, sexual dimorphism and male heterogamety are a general rule in Decapoda (Makino, 1951· Charniaux-Cotton, 1960). In several Decapoda species the XX/XY, XX/XO, and XXX/XX sex chromosome mechanisms have been reported (Makino, 1951), and it is likely that H.americanus is not an exception in this respect. The sex chromosomes of P.cornutus are of the XX/XY type, and those of L.maderea are of the XX/XO type (Makino, 1951). In both species the male is the heterogametic sex. The sex chromosomes of D.punctata are unknown, but conceivably, it is also of the XX/XO type, since this sex chromosome mechanism is predominant among Orthoptera (Makino, 1951). It therefore appears that crossreactivity between the mammalian H-Y antigen and the invertebrate antigen is associated with the heterogametic sex of the lobster and the Coleopteran beetle but with the homogametic sex of the Orthopteran species. In the latter case, heterogamety of the male is due to the lack of a second X, but not to the presence of a hemizygous sex chromosome (Y or W). This difference might be of significance; while the operation of a sex specific gene in the sex which carries a hemizygous chromosome will regularly be dependent on the presence of a gene (structural or regulatory) linked to that chromosome, in the case of the XX/XO mechanism, one must assume a quantitative effect which could depend (as in the cases of Drosophila sex determination) on the balance with other autosomes. Alternatively, the two X chromosomes have a differential region which does not undergo crossing over. In this case one X chromosome(X^i) may carry an inducer gene while the other may carry the structural gene(X^s). Females of the X^iX^s combination express the sex specific product while X^iO and X^sO males do not. X^iX^i and X^sX^s females which do not express the antigen are eliminated at early stages or may live at equilibrium.

While the role of the mammalian H-Y antigen in testicular organogenesis has been established (Ohno, 1979), the possible role of the crossreactive H-Y antigen in birds, fish, and amphibia is yet to be investigated. A connection between the evolution of sex specific cellular antigen and the evolution of cell-cell recognition systems has previously been suggested and discussed (Monroy and Rosati, 1979). It is possible that the original role of an H-Y-like antigen in metazoa was in sexual interactions or self recognition between male and female organisms (prevention of self fertilization). Such cellular recognition mechanisms are common among lower colonial marine forms as well as among protista and flowering plants (Crandall, 1977: Monroy and Rosati, 1979; Katz and Skidmore, 1978; Theodor, 1920: Burnet, 1921). While "self - non-self" recognition appears to be a fundamental biologic process concerned with control of many developmental and differentiation events (Katz and Skidmore, 1978), self antigens which are involved in sexual determination must comprise a sub-specificity associated with only one sex within each species. It is therefore conceivable that as a sex-specific cell surface molecule, H-Y antigen had the evolutionary advantage of becoming the major candidate in the primary determination of the mammalian gonad. If this hypothesis is correct, It may be possible to identify an H-Y like molecule in even more primitive eucaryotes (e.g. protista) , and to correlate the evolution of the genetic control on sex determination , the role of the H-Y-like antigen in sexuality and the molecular changes in its structure.

Summary

A sex specific antigen which crossreacts with the mammalian H-Y antigen has been identified on the cell surface of hemocytes from the lobster (Homarus americanus) and the gonadal cells of three insect species. The hemocytes from the male lobster, the testicular cells from the male beetle (P.cornutus) and the ovarian cells from two Orthopteran species (L.maderae and D.punctata) specifically absorbed H-Y antibodies. The specificity of H-Y antibody absorptions by cells from only one sex, suggests that an ancestral H-Y-like antigen may be present in invertebrates which could be engaged in sexual (cellular) recognition events.

CHAPTER III

EXPRESSION OF H-Y ANTIGEN IN THE GUPPY (LEBISTES RETICULATUS) *

* In press in Differentiation. Done as a joint study by A.Shalev and E.Huebner, from the Department of Zoology, University of Manitoba.

The H-Y antigen is a unique example of a histocompatibility antigen with a defined ontogenic function (testicular organogenesis) and wide phylogenic distribution. The role of H-Y antigen as a organized of the undifferentiated mammalian gonad into a testis has been inferred from cases of 'Nature errors' in sex determination and from in vitro experiments: manipulating 'testis-like' or 'ovary-like' structures by the use of anti H-Y sera or soluble H-Y antigen fluid (Zenzes et al., 1978a, 1978b: Ohno, 1979: Wachtel and Ohno (1979): Nagai et al., 1979). The evolutionary conservation of H-Y antigen in vertebrates has been shown by the fact that absorption of the anti H-Y sera with cells from one sex (usually the heterogametic one) resulted in a drop in sperm cytotoxicity (Wachtel et al., 1975) or hemagglutination titer (Shalev et al., 1978). In the present study we used the protein-A rosette technique , previously described by Koo and Goldberg (1978), to test for presence of a crossreactive H-Y antigen in the guppy (Lebistes reticulatus). As shown (Table 1), the absorption of rat anti H-Y sera by the pooled cells obtained from the male guppy, but not the female, resulted in a significant drop in rosette formation by rat H-Y antibody, equivalent to the drop observed following the absorption with male rat splenocytes. Thus , indicating that Lebistes is endowed with a male specific antigen which crossreacts with the mammalian H-Y antigen.

Presence of a male specific histocompatibility antigen in fish was first suggested by scale transplantation in an inbred population of Xiphophorus maculatus (Miller, 1962). This early observation has recently been substantiated by an in vitro study (S.S.Wachtel, personal communication). Whereas in a previous experiment (Shalev et

Table 1

Rosette formation with male rat (Lewis) lymphocytes following absorption of anti H-Y serum with cells from male and female fish and rat.

	% rosette formation (mean ± S.E.)
Absorbed with male cells (rat)*	18.7±3.0
Absorbed with female cells(rat)*	29.2±3.5
Absorbed with male cells (guppy)	21.1±2.0
Absorbed with female cells (guppy)	31.8±2.1
No absorption (positive control)	30.8±2.6
No antiserum (negative control)	6.3±1.3

The figures represent 8 separate tests (* or 3 tests) using coded samples derived from male and female fish and rat. Anti H-Y serum (1:4 dilution) was absorbed with an equal volume (30 µl) of cells derived from the rat spleens or from pooled fish tissues. The liver, heart, spleen, and gonads of two fish of each sex were mechanically macerated in Eagle's basal medium (Gibco, #230) and rat spleens were macerated into phosphate buffered saline. The cells were washed once in 10 ml. of the same media prior to absorption.

Anti H-Y serum was produced in Lewis rats by 10 injections of 2 x 10^6 male splenocytes at 2-3 days intervals which was followed by a booster injection of 1 x 10^7 cells 6 weeks later. In previous tests this antiserum produced 300 % rosette formation with male lymphocytes as compared to female lymphocytes.

The results obtained following absorption with male cells are significantly different ($p<0.01$) from those obtained following absorption with female cells for each species respectively.

al, 1978) using blood cells from the rainbow trout (Salmo gairdneri) it was not possible to clearly determine which sex, if at all, was H-Y positive, in the present experiment we identified the sex of the cells donor (coded) with no difficulty after each test.

It is established that the male guppy is the heterogametic sex (see Haskins et al., 1970; Makino, 1951: White, 1973), which is indeed in accord with the general findings of association between H-Y antigen and the heterogametic sex (Wachtel et al.,1975: Shalev et al., 1978)). Extensive studies by Winge and his associates on the guppy (see White, 1973) have provided that by transfer of sex determining genes to the autosomes the heterogametic sex can be reversed from the male to the female. This and other evidence led White (1973) to conclude that sex determination mechanisms in cypridont fish are in a primitive and unstable state. Unlike mammals, in which sex determination is simplified by the existence of major sex determining genes, the situation in lower vertebrates might be more complicated (Ohno, 1979). Demonstration of sex chromosomes as well as stable sex ratios are strong indicators for genetic control on sex determinatin in fish. However, the ease at which fish can be sex reversed by hormonal and environmental manipulations (White, 1973: Chan, 1970: Schreck, 1974: Reinboth, 1975) suggests a significant role for non-genetic components in determining the gonadal sex. The genetic component can therefore either interact with or be controlled by the external factors. If H-Y antigen is responsible for gonadal organogenesis in fish then one should expect that factors which will turn an ovary into a fertile testis (assuming the XX/XY sex chromosomes constitution) will also induce expression of H-Y antigen. Indeed , in the higher class Aves in which the sex chromosome mechanism ZZ/ZW is predominant and the female

is H-Y positive, male chicken embryos were shown to express H-Y (H-W) antigen after oestradiol treatement (Muller et al., 1979). Can environmental factors simulate the hormonal effect in fish to induce expression of H-Y antigen ? The possiblity that H-Y antigen is not relevant to sex determination in fish might also be considered. Nevertheless, since a nonesence presence of the antigen on the surface of cell on only one sex is difficult to comprehend, we suggest that the H-Y crossreactive antigen in fish might have a limited role in the differentiation of the gonad. This can possibly represent a transitional evolutionary stage before H-Y antigen acquired its major role in mammals.

While the serological experiments like the present one explore the conservation of a unique (H-Y) antigen and the evolution of the genetic control on sex determination ,recent results from our laboratory indicate that an H-Y crossreactive antigen is present in invertebrate species(Chapter II). This may imply that the original evolutionary function of the ancestral H-Y antigen could have been in sexual cell-cell recognition events such as the known role of the mating antigens in protista (Crandall, 1977).

Summary

Expression of a mammalian crossreactive H-Y antigen on the surface of cells derived from the male guppy (Lebistes reticulatus) is demonstrated. This finding further establishes the evolutionary conservation of H-Y antigen among lower vertebrates and provides a basis for speculation on the possible evolutionary association between

H-Y antigen and sex determination.

Note in Proof

Following the acceptance for publication of this communication, two papers on the presence of H-Y antigen in fish were published in this Journal (Pechan *et al.*, Differentiation 14, 189, 1979 and Muller and Wolf, Differentiation 14, 185, 1979). The presence of H-Y antigen in several teleostean fish including *Xiphophorus hilleri*, *X. maculatus* and *Lebistes reticulatus* was demonstrated.

It is of interest to note that in accord with a previous report (Shalev *et al.*, 1978), Muller and Wolf (1979) were unable to clearly identify one sex of *Salmo gairdneri* which was H-Y positive; though their results indicate that ovarian cells absorbed more H-Y antibodies. The authors considered the possibility that the absorption by both sexes could have been the result of a non-specific reaction between rat immunoglobulins and fish xenogenic antigens (such possibility is naturally excluded when a sex specific or anti H-Y specific reaction is observed). In light of our recent report (Shalev *et al.*, in press) on the presence of an H-Y crossreactive antigen in invertebrates, the suggestion by Muller and Wolf (1979) that H-Y antigen was originally shared by both sexes should be reexamined.

CHAPTER IV

46,XY GONADAL DYSGENESIS AND H-Y ANTIGEN *

This study was carried out in collaboration with Dr's F.I.Reyes, J.Blankstein, J.S.D.Winter and C.Faiman, from the Department of Pediatrics, University of Manitoba.

It is established that individuals with XY gonadal dysgenesis fail to develop testes in spite of having a normal 46,XY karyotype. Recently German et al. (1978) described a pedigree in which this disorder appeared to segregate as an X-linked recessive characteristic; in this pedigree three different Y chromosomes (from three different fathers) were represented in affected individuals. This evidence points to the existence of a gene on the X chromosome which acts to control the expression of the Y-linked testis-determining gene.

Since several lines of evidence suggest that the H-Y antigen of normal male cells represents the product of the testis-determining gene (Wachtel, 1977; Ohno et al., 1978), it would be of interest to determine whether individuals with 46,XY gonadal dysgenesis normally express H-Y antigen. To date the results of such tests are variable; the patient of Dorus et al. (1977) was H-Y positive, whereas three patients studied by Ghosh et al. (1978) were H-Y negative. It is therefore not clear whether the defect in 46,XY gonadal dysgenesis lies in the expression of the H-Y membrane moiety or in another gene which may be responsible for the action of H-Y antigen as the testis inducing factor.

We have examined H-Y antigen in two lines of cells from a 16-year old patient with XY gonadal dysgenesis who presented with normal female external genitalia and sexual infantilism. There was no family history of a similar disorder and no consanguinity. Serum levels of follicle stimulating hormone, luteinizing hormone, testosterone and estradiol concentrations were in the castrate range. Laparotomy disclosed infantile female internal genitalia and streak gonads;

Table I.

Rosette Formation by Mouse Male Lymphocytes Following Incubation with Absorbed H-Y antiserum.*

	Percent Rosettes (of ≥200 cells counted)			
	Run 1	Run 2	Run 3	Run 4
No absorption of H-Y antiserum	18.3	20.4	15.8	10.2
Absorption with female fetal fibroblasts	14.7	16.9	16.7	8.6
Absorption with male fetal fibroblasts**	10.2	9.1	6.6	1.8
Absorption with gonadoblastoma cells of patient**	7.4	7.8	6.2	2.2
Absorption with skin cells of patient**	9.2	10.0	10.1	3.2
Control (no H-Y antiserum)	4.7	5.0	3.2	0.8

*H-Y antiserum was produced in C57Bl/6 mice and showed high specificity in binding to male lymph node lymphocytes as compared to female lymphocytes (34.4% and 21.4% vs. 9% and 4% rosettes, respectively). Mouse male lymphocytes were incubated on ice with H-Y antiserum (absorbed or unabsorbed) and spun together with staphylococcal Protein A coated sheep red blood cells. Runs 3 and 4 were performed in double blind fashion.

**The results following absorption with male fibroblasts and with the patient's gonadal and skin cells are significantly different ($p<0.01$, paired t-test of 4 runs) from the tests with no absorption and absorption with female cells, but are not different from each other.

histological examination of the latter confirmed bilateral gonadal streaks with gonadoblastoma and dysgerminoma. Cytogenetic analysis of blood lymphocytes, skin fibroblasts and gonadoblastoma cells showed a 46,XY chromosome complement (50 cells). The karyotypes of three healthy sisters were 46,XX.

Cultured cells from the patient's skin and gonadoblastoma were tested for H-Y antigen by a modified protein-A rosette technique (Part 1 and Chapter V) As shown in Table 1, absorption of H-Y antiserum by these cells caused a significant ($p<0.01$) reduction in rosette formation, equal to that caused by fetal male fibroblasts. This indicates that the patient's cells were H-Y antigen positive, and that the density of H-Y antigen on their surface was similar to that of normal male fibroblasts.

These data, and those of Dorus *et al.* (1977), indicate that in some individuals with 46,XY gonadal dysgenesis H-Y antigen is produced. Possibly in such cases the defect in testis formation occurs at the level of the gonadal specific receptor for H-Y antigen (Zenzes *et al.*, 1978a) or at a locus coding for a product which acts at a more distal step of testicular organogenesis.

The three cases (two of them siblings) of H-Y negative, 46,XY gonadal dysgenesis described by Ghosh *et al.* (1978) would seem to represent a different mechanism for the lack of testis development: the defect in these patients may lie in the expression of H-Y antigen itself. Such a mechanism would be analogous to the situation in the wood-lemming (*Myopus schistocolor*), in which affected XY females are fertile, H-Y negative and seem to carry an X-linked mutation (Wachtel *et al.*, 1976).

If further examination of more cases of 46,XY gonadal dysgenesis discloses the existence of both H-Y positive and H-Y negative subtypes, this would suggest that normal testis formation requires the presence not only of an X-linked regulatory gene operating in conjuction with the H-Y gene, but also of the gene(s) which controls the expression of the gonadal receptor for H-Y antigen or of a yet undetermined product which functions at a later stage of testicular organogenesis.

Addendum

While this study was completed in February 1978, it was not submitted for publication until March 1979 because of unfortunate loss in the mail. In the meantime a study on H-Y antigen in 12 cases of XY gonadal dysgenesis was published (Wolf et al.,1979). The author found that three of them were H-Y negative and the rest were H-Y positive. The author proposed that a mutation in the gonad specific receptors for H-Y antigen might be responsible for this condition in the cases in which H-Y antigen is expressed.

CHAPTER V

IMMUNOGENETICS OF SEX DETERMINATION IN THE POLLED GOAT *

* Submitted for publication as a joint study by A.Shalev, Dr.R.V Short from the MRC Unit of Reproductive Biology, 37 Chalmers St., Edinburgh, Scotland, and Dr.J.L.Hamerton from the Department of Anatomy (Human Genetics), University of Manitoba.

Introduction

Genetically female (XX) goats homozygous for the autosomal dominant P (Polled) gene have been shown to be male pseudohermaphrodytes (Hamerton et al., 1969: Short, 1972), although the mechanism of action of the P locus is uncertain. Recent evidence suggests a role for H-Y antigen as a testis-organizing agent in mammals (Wachtel and Ohno, 1979: Ohno, 1979: Zenzes et al.,1978: Muller et al., 1978: Short, 1979). A strict association between the presence of the H-Y antigen and testicular development, even in the apparent absence of a Y chromosome has also been established (Bennett et al., 1977: Forobosco et al., 1978: Nagai and Ohno, 1977: Selden et al., 1978: Wachtel et al., 1976: de la Chapelle et al., 1978: Wachtel et al., 1978). Thus , despite a report of the mapping of the H-Y locus on the Y chromosome (Koo et al.,1977) the possibility remains that the structural H-Y locus (or loci) can be located on a chromosome other than the Y which in turn may carry a regulatory gene or genes for H-Y antigen expression.

In the present study the expression of H-Y antigen on cultured fibroblasts from a family of Saanen goats, carriers of the P gene, was investigated.

Material and Methods

Animals. One to seven year old Saanen goats were bred as described in Figure 1. Two of the males (F and G) were castrated 3 days after birth. Intersex D was mildly masculinized with no palpable testes, while intersex E had palpable scrotal testes. Upon autopsy following

the immunological examination , it was found that both intersex goats (D,E) had a pair of small intra-abdominal testes with well-developed epididymis.

Cultures. Skin biopsies were taken under local anesthetic, coded, and shipped by air from London to Winnipeg. Primary cultures were established in Winnipeg by standard methods and grown in McCoy's 5A (Modified) medium (Gibco) enriched with 10% fetal calf serum (Mc10Fcs). Cell cultures were grown in monolayers for 10 to 15 transfers and harvested by trypsinization with 0.05% trypsin until detached (10-15 min.). The cells were then washed twice in phosphate buffered saline (PBS) before being used for serolgical testing. All testing was carried out on coded samples and the code was not broken until the serological tests were complete and had been interpreted.

Serology. H-Y antibodies were produced in females of inbred DA and Lewis rat strains by 10 intraperitoneal injections of 2-3 x 10^6 male spleen lymphocytes at 2-3 day intervals, followed by a single booster of 1 x 10^7 cells given 6 weeks later. Animals were bled 7 days later and the sera tested individually for anti-H-Y activity. High specificity antisera were pooled from 3 DA or Lewis rats (Table 2) and used for the tests as described below.

The procedure for H-Y typing was modified from Koo and Goldberg (1978). Briefly, staphylococcal protein A (PA) (0.1 ml. containing 1 mg/ml) is conjugated to 0.1 ml. of packed sheep red blood cells (SRBC) by dropwise addition of 1 ml. 0.01% $CrCl_3$ (pH 5.0). The suspension is left for about 5 min. at room temperature and the coupled SRBC (PA-SRBC) are washed with PBS. Male and female lymphocytes are preincubated on ice with anti-H-Y sera and washed (x3) with ice-cold

PBS. The lymphocytes are then centrifuged together with PA-SRBC at 180 x g for 10 min. and the pellet is left for a further 60 min. at room temperature. Rosette formation (as a result of binding between the PA and Fc portion of IgG) is counted in about 200 lymphocytes following the addition of a vital colorant (Crystal Violet 1%).

Percent rosette formation with male rat cells was measured following the absorption of H-Y antisera by an equal volume (20 µl.) of goat cells. The inhibition of rosette formation was measured by incubation of 20 µl antisera with an equal volume of culture medium from the goat cells. The final dilutions of H-Y antisera in all tests were identical (1/4). Tests were done consecutively.

Statistics. Percent inhibition (Table 1) was determined by the following formula 100x(%rosettes positive control-%rosettes test)/%rosettes positive control. Pairs of values from different animals in every test were compared in a pairwise fashion (paired t-test) for 8 absorption tests and 6 media inhibition tests (Table 1).

Results

Chromosome Analysis : No Y chromosome was observed in 50 metaphase spreads from the two intersexes (D,E) or from the two females (B,C). The three male animals had a Y chromosome present in each cell.

Serology : Eight absorption tests were performed on the cultured fibroblasts. Six inhibition tests were carried out using culture medium in which the fibroblasts had been grown for 72-80 hours. The results showed that the only H-Y negative animal was the heterozygous

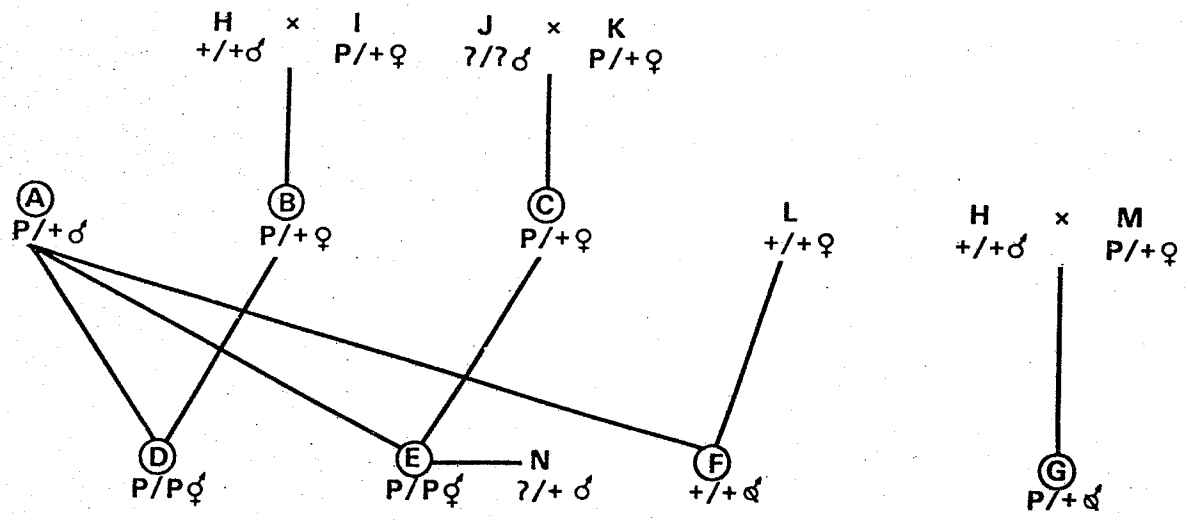
polled female (C), since its cells did not shed H-Y antigen nor did they absorb H-Y antibody (Figure 2, Table 1). In contrast, the fibroblasts of a second polled female (B) apparently shed H-Y antigen and were capable of absorbing H-Y antibody (Figure 2). Statistically, the results of the tests with this female (B) were not different from other males or from female C , but they differed significantly from the controls (Table 1). Thus , although less positive than the males, this female was apparently H-Y positive. The cells from the two intersex goats (D,E) showed similar expression of H-Y antigen: one intersex (D), clearly had a reduced capacity to absorb H-Y antibody when compared to the castrated males (F,G): however, the shedding of H-Y antigen into the medium by cells from animal D was not different from that of other males (Figure 2, Table 1). These results suggest that the density of H-Y antigen on the cell surface of cells derived from the intersex goats may be lower than the density found on the cells of normal males. The sire (A) of the intersexes (Figure 1) was clearly H-Y positive. The cells from this animal appeared to be only weakly positive by absorption and not statistically different from the males (Table 1), but inhibition tests showed it to shed high levels of H-Y antigen (Figure 2).

Discussion

According to the "genic balance" theory (such as in Drosophila), sex is determined by the balance between male and female determining genes which reside on the X chromosome and autosomes. However, in mammals major genes for sex determination must be postulated (Ohno,

Figure 1

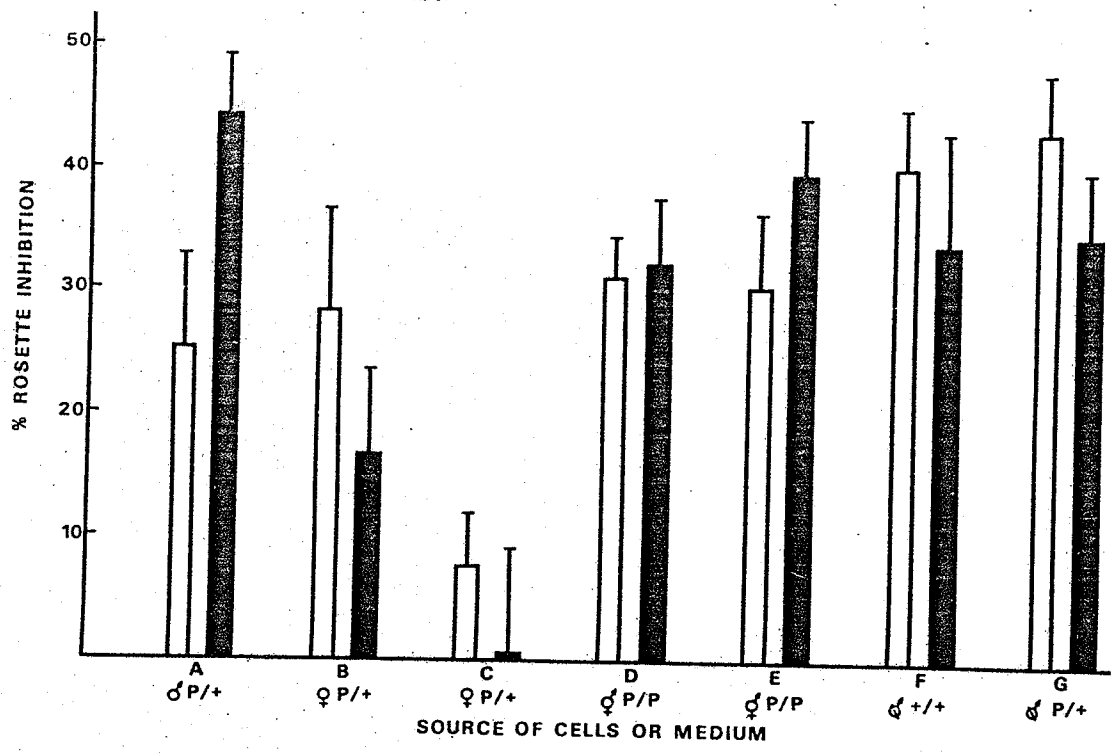
Pedigree of the experimental goats



Encircled animals were tested for presence of H-Y antigen. The genotype of the animals was designated as follows: $\pm/\pm$ -Horned , $P/\pm$-polled heterozygous and P/P -polled intersex.

Figure 2

Inhibition of rosette formation by cellular absorption and incubation with culture media from the experimental goats.



The mean±S.E. of rosette inhibition for 8 absorption tests and 6 medium inhibition tests is presented by the white and dark bars respectively. Rosette formation was reduced following absorption of H-Y antibodies (1:4 dilution) with an equal volume (20 µl) of cultured cells or by incubating an equal volume (20 µl) of media from 72-80 hour cultures with H-Y antibodies. For calculation of % inhibition see below. The values of the positive (no absorption or inhibition) and negative (no antiserum) controls were 21.7% and 3.9% respectively for the 8 absorption tests and 23.7% and 4.2% for the 6 inhibition tests respectively.

Table 1

Statistical evaluation of absorption and inhibition of rat H-Y antibodies by cells and media of fibroblast cultures from the goats. (results presented in Figure 2).

ANIMAL

Compared to animal	A ♂ P/+	B ♀ P/+	C ♀ P/+	D ♂ P/P	E ♂ P/P	F ♂ +/+	G ♂ P/+	Unabsorbed control
A							S	0.05
B	0.05		0.05					0.01
C	0.01			0.01	0.01	0.01	0.01	
D	0.05		0.05			0.05	0.05	0.01
E			0.05				0.05	0.01
F			0.05					0.01
G	0.05		0.05					0.01
Unabsorbed control	0.01	0.05		0.01	0.01	0.05	0.01	

MEDIUM INHIBITION

CELLULAR ABSORPTION

The upper right half of the table represents the statistical results of 8 absorption tests and the lower left half represents the analysis of 6 inhibition tests. Blank squares denote non-significance at $\alpha=0.1$ level otherwise P is lower than the level indicated (0.05 or 0.01). S signifies significant difference at $P<0.1$ level when the same combination by the medium inhibition test was significant at $P<0.05$ level.

Table 2

The specificity of the rat H-Y antisera used for testing

Source of antisera (strain)	Absorption	% rosette with male (or female) lymphocytes at various dilutions. 1:2	1:4	1:8	1:16
Lewis	____	28.5(10.4)	35.6(11.1)	30.8(9.6)	18.4(7.1)
	male spleen cells	13.4	14.6	15.8	10.3
	female spleen cells	27.9	33.7	28.1	15.7
DA	____	20.6(7.8)	23.4(8.9)	22.5(9.0)	13.8(6.2)
	male spleen cells	11.3	11.1	15.4	8.5
	female spleen cells	19.9	21.0	20.2	11.3
Normal (DA) serum	____	6.3	5.9	4.0	2.8

Each antiserum was pooled from 3 Lewis or 3 DA animals which have previously proved to have a good anti H-Y response by individual testing. Unabsorbed antisera were tested on both male and female lymph-node lymphocytes (same strain) and absorbed antisera were tested only with male lymphocytes.

1967; Hamerton, 1968; Ohno, 1979) and sex determination is known to be of the Y-dominant (or epistatic) type: that is, presence of one or more Y chromosomes usually determines a masculine gonad irrespective of the number of X chromosomes. The genetic mode for intersexuality in the goat has been established to be autosomal recessive and is invariably associated with the autosomal dominant gene for polledness (P). Thus, it was reasoned that a gene for sex determination is closely linked to P or that P is highly pleitropic with a sex-limited recessive effect on sex determination, leading to partial or total sex reversal of XX females (Short 1972).

In a recent study , using the sperm cytotoxicity test, Wachtel et al., (1978) also found that two intersex goats were H-Y^{+}. Statistical analysis indicated that the intersexes were less positive than a normal male and suggested that a heterozygous (P/+) female might be weakly positive. The present results are in close agreement with those of Wachtel et al., (1978), Nevertheless, we will propose an alternative hypothesis for the abnormal transmission of H-Y antigen in the polled goat. Our observations suggest that: (a) the density of H-Y antigen on the cell surface of homozygous (P/P) pseudohermaphrodite goats is lower than that on the cell surface of normal (+/+) or heterozygous (P/+) males; (b) one heterozygous normal female (B) but not another (C) appeared to be H-Y positive; (c) cells from one heterozygous male (A) seemed to shed more H-Y antigen into the medium but had a lower capacity to absorb H-Y antibody. Thus, within the limits of sensitivity of the methodology used, our results suggest that animals of the same sex and genotype may differ in H-Y antigen density or shedding capacity.

The exact relations between the P gene (or the P-linked sex reversal gene) and the H-Y locus are of interest. While it is difficult to envisage that the gene (P) for polledness also codes for H-Y antigen, it appears more likely that the sex reversal gene closely linked to P coded for a subnormal dose of H-Y antigen (Wachtel et al., 1978). If the linkage between P and the sex reversal (H-Y) gene are so close that crossing over between the two cannot be detected, it might be expected that polled animals (including females) should invariably express H-Y antigen. Accordingly, XY, P/+ and XY, P/P males should maintain a higher density of H-Y antigen on the cell surface as a result of extra copies of H-Y genes. If crossing over does, however, occur between the two presumed genes, it should be possible to identify horned pseudohermaphrodite goats as well as fertile females homozygous for P. Our data, within the limitation of the number of animals examined do not support dosage expression of H-Y antigen as a function of the number of P genes since no difference was found in the ability of cells from a normal male (+/+) as compared to a polled one (P/+) either to absorb or to inhibit H-Y antibody (Figure 2, Table 1). Furthermore, one polled and fertile female but not the other expressed H-Y antigen. Nonetheless , the two intersex goats had a reduced capacity to absorb H-Y antibody (Figure 2, Table 1). We suggest that extreme variability in gonadal differentiation frequently seen among intersex goats (Hamerton et al., 1969;Short, 1972) might be explained by variability in H-Y dosage. This is possible only if one assumes dosage polymorphism in the independent P linked sex reversal (H-Y) gene among intersex goats. Other possible explanations for the extreme variability of gonadal differentiation among intersex goats include in utero maternal effects such as maternally induced and transmitted H-Y

antibodies association of other histocompatibility antigens with H-Y antigen, hormonal influence and biological randomness of the timing of cellular differentiation.

The hypothesis that a Y-autosome or Y-X translocation may be responsible for the expression of H-Y antigen in the absence of a Y chromosome in a significant number of species (see introduction) seems remote in view of the lack of any appropriate cytological evidence. Nonetheless, Y-linkage, X-linkage or even autosomal location of the H-Y gene(s) might occur in some species. Indeed, in the Mole-Vole (Ellobius lutescens) in which only one X chromosome is found in both males and females , the expression of H-Y antigen in males depends on an X-linked or autosomal H-Y gene (Nagai and Ohno, 1977). In the chicken, expression of H-Y is apparently not W-linked as male chicken embryos (ZZ) gonad can hormonally be induced to express H-W antigen (Muller et al., 1979). On the other hand, strong evidence suggests the Y-linkage of the H-Y gene in the human (Koo et al., 1977; Wachtel et al., 1975).

Hamerton (1968) first suggested that the Y chromosome of mammals might carry a regulatory gene for X-linked sex determining gene(s). In mammals with an XY/XX sex determining mechanism, it is very difficult to distinguish experimentally between this hypothesis and the concept of Y-linkage of H-Y gene which is controlled by a regulatory gene on the X-chromosome. The first hypothesis however, would predict presence of the structural H-Y gene(s) on the X in both males and females . Hence, the expression of H-Y antigen in some XX goats as well as in the mole voles could be explained on the basis of an autosomal mutation which mimics the role of the Y-linked regulatory gene. The

recessive mode of action of the sex reversal gene in the goat and the quantitative differences in H-Y antigen cell surface density observed among the different genotypes of polled goats are not easily explained by this hypothesis, but can be reconciled if one assumes quantitation of the inducer loci and substance rather that the H-Y structural gene(s). Such a situation seems, however, rather complicated.

Summary

Using the protein A rosette technique, it was found that the gene for polledness (P) in goats is associated with the presence of H-Y antigen on the cell surface of cultured fibroblasts. Two XX intersex goats (P/P) were found to be H-Y$^+$ and one heterozygous (P/+) normal XX female was also found to express H-Y antigen at a low level. The expression of H-Y antigen by intersex goats was found to be lower than that of normal XY males. The statistical analysis suggests that animals of the same genotype and sex might differ in the density of H-Y antigen on their cell surface, which might explain variability in primary sex determination among intersex goats. The association between the gene for sex reversal and H-Y antigen is discussed in relation to the location of the H-Y gene(s).

PART 3

H-Y ANTIGEN AND FETAL-MATERNAL INTERACTIONS

CHAPTER VI

HEPATITIS B VIRUS, SEX RATIO AND THE H-Y ANTIGEN *

* In press in J. Clin. Lab. Immunol. Done as a joint study by A.Shalev and C.K.Hannan, from the Department of Medical Microbiology, University of Manitoba.

Introduction

Our interest in the possible relation between disease and sex-ratio was stimulated by a chance finding in which a control group of experimental mice displayed an unusual sex-ratio associated with apparent infectious disease.

In a recent study of a Greek population, Drew et al. (1978, 1978a) reported that parental response to infection by Hepatitis B Virus affected the secondary sex-ratio (number of males per 100 females at birth) of the offspring. The chronic Hepatitis B surface Antigen (HBsAg) carrier state in either parent was significantly correlated with higher than normal sex-ratio, whereas the presence of maternal (but not paternal) anti-HBs correlated with a sex-ratio which was lower than normal. Drawing on other observations, including the higher prevalence of the chronic HBsAg carrier state in males than in females, and differential survival rates of kidney transplants from male donors in HBsAg positive and anti-HBs positive recipients (Drew et al., 1978, 1978a), Drew and her colleagues interpreted these findings as suggesting the existence of a crossreactivity between HBsAg and a male-associated antigen. If correct, this interpretation could have significant implications in studies of both Hepatitis B and sex-related antigens. However, even if disputed, such studies provide insight into factors which affect sex-ratio.

In this communication, we report that we have been unable to find any evidence for crossreactivity between HBsAg and H-Y antigen, the

only known male specific antigen (Wachtel, 1977). Some additional considerations which relate to Hepatitis B infection and sex-ratio are discussed in the light if this finding.

Material and Methods

Anti-HBs Sera

Two human sera with high levels of anti-HBs were used. One was obtained from a male hemophiliac (code 6055C) and the second from a normal female blood donor (code 2350G); anti-HBs titres measured by passive hemagglutination were 1:64,000 and 1:16,000, respectively.

Subtype specific anti-HBs/ad and anti-HBs/ay were produced in male guinea pigs by immunization with highly purified HBsAg of each subtype (see following paragraph) following a protocol described earlier (Hannan and Malyska, 1973). Antibody levels in pooled guinea pig sera were determined by passive hemagglutination of red blood cells sensitized with HBsAg of the corresponding subtype; the antibody titres were 1:5,120,000 (anti-HBs/ad) and 1:256,000 (anti-HBs/ay). The monospecific nature of the anti-d or anti-y component of each serum was confirmed by Ouchterlony immunodiffusion testing with standard HBsAg preparations of known subtype (LeBouvier, 1971; LeBouvier and Williams, personal communication).

HbsAg of known subtype used for immunization of guinea pigs originated from two chronic asymptomatic carriers: HBsAg/ay was provided by a female donor and HBsAg/ad by a male. The antigen was extracted from defibrinated plasma and purified by a 3-step

ultracentrifugation procedure (Hannan and Malyska, 1972) comprising two consecutive isopycnic bandings un discontinuos cesium chloride gradients followed by rate zonal sedimentation in a linear sucrose gradient.

Anti-H-Y Sera

Female C57Bl/6 mice were inoculated repeatedly with male spleen cells of the same strain (15 intraperitoneal injections of 3×10^6 cells in 0.1 ml.). The three anti-H-Y sera used in the investigation had the following immune specificities to form rosette with male and female mouse lymphocytes, respectively: 24.5% vs. 3.0%; 21.6% vs. 4.3% and 34.4% vs. 9.0%. The reactivity of these mouse antisera with human H-Y antigen had been tested previously and resulted in 30%-80% reduction in rosette formation following absorption with human male cells as compared to female cells (4 tests).

Serological Procedures for Anti-HBs and HBsAg

Passive hemagglutination (PHA) for detection and titration of anti-HBs was performed as described by Vyas and Shulman (1970). Erythrocytes sensitized with either HBsAg/adw or HBsAg/ayw and control erythrocytes were obtained from Electro-Nucleonics, Inc., Bethesda, Md. Unless otherwise stated, all sera were screened in 1:8 dilution with RBC sensitized with HBsAg/adw only and with control (unsensitized) RBC). Specimens which were presumptively positive in the screen test were confirmed by titration from an initial 1:4 dilution with both sensitized and control RBC. Positive and negative

controls were included in each test.

All serum samples were tested for the presence of HBsAg by solid-phase radioimmunoassay (RIA) (Austria-II, Abbott Laboratories, North Chicago, Ill.) using procedure "B", i.e., first stage incubation overnight at 22°C, and second stage incubation for 1 hour at 45°C (Ling and Overby, 1972). Samples giving positive results in the screen test (sample cpm > 2.1 x negative control mean cpm) are re-tested by specific neutralization with anti-HBs to confirm the presence of HBsAg.

Protein A Rosette Technique

The procedure for H-Y typing is described in the Introduction (Part 1) and in Chapter V. The identical rosette technique was used for examining rosette formation by guinea-pig antisera and leukocytes.

Results

The sharing of antigenic determinants between HBsAg and the male-specific H-Y antigen should be detectable by reciprocal tests of reactivity between each antigen and the heterologous antibody. However, when sex-specific antigenic differences may exist - as in this instance - it is conceivable that the sex of the antibody donor could be a factor in the interpretation of the results of such tests. Accordingly, the reactivity of anti-HBs of human male and female origin with male and female rat (DA strain) lymphocytes was tested by the PA rosette technique. As shown in Table 1, whereas a rather high level of rosette formation occured with the unabsorbed anti-HBs sera,

Table 1

Rosette formation by human anti-HBs sera with male and female rat lymphocytes prior to and following absorption with female cells

antiserum[a]	% rosettes with unabsorbed antiserum		% rosettes with absorbed antiserum[b]	
	Male cells	Female cells	Male cells	Female cells
6055C	63.6	80.4	7.8	8.3
2350G	40.4	32.5	8.4	7.2
anti H-Y	30.7	8.3	25.6	5.2
nil	4.1	3.3		

[a] Notations for antisera codes are given in Materials and Methods. Anti-H-Y antiserum had previous rosette formation capacity of 24.5% and 3% with male and female cells respectively.

[b] Rat (DA) lymph-node lymphocytes were used for reaction in all cases. Absorption (x3) was done on ice with equal volumes of antiserum and packed female spleen cells.

Table 2

Rosette formation by human male and female leukocytes following incubation with guinea pig anti-HBs sera which have been absorbed by female cells[a]

Source of leukocytes	% rosettes with anti-HBs/ad	% rosettes with anti-HBs/ay	% rosettes with control serum[b]
male 1	7.4	9.1	5.9
male 2	5.3	4.5	4.0
male 3	2.2	3.0	3.1
male 4	8.6	6.9	8.9
male 5	9.3	10.1	6.2
female 1	5.7	6.4	5.8
female 2	9.2	8.7	6.9

[a] For notation of the anti-HBs sera see Materials and Methods. Absorption of sera with equal volumes of pooled leukocytes from 6 different unknown females was done prior to rosette testing. All donors were hematologically normal and all were negative for HBsAg and anti-HBs.

[b] A HBsAg and anti-HBs negative human male serum was used as negative control. Because of non-specific crossreactivity it was not possible to use the heterologous anti H-Y anti-serum as positive control; however, to confirm the effectiveness of the test a sample of human (male) leukocytes was tested with anti-human thymus serum (Shalev et al. 1977) which gave 76.6% rosette formation.

Table 3

Reactivity of human and guinea pig anti-HBs sera before and after absorption with human male or female leukocytes

Anti-HBs serum[a]	1/anti-HBs titre (PHA)		
	Unabsorbed	Absorbed with male cells[b]	Absorbed with female cells[b]
6055C (human)	64,000	32,000	32,000
2350G (human)	16,000	32,000	32,000
anti-ad (g. pig)	2,560,000	5,120,000	5,120,000
anti-ay (g. pig)	256,000	1,024,000	512.000

a See Materials and Methods for details.

b Each antiserum was absorbed twice (30 mins. on ice) with an equal volume of pooled leukocytes obtained from 2 male or 2 female human donors. The serum of each donor was negative for HBsAg and anti-HBs.

only background levels were detected following absorption with female rat cells. By contrast , the anti-H-Y serum formed rosettes with male rat cells to a significantly greater degree than with female rat cells, whether absorbed with female cells or not.

In the second experiment, the reactivity of male guinea pig anti-HBs sera of defined antibody content (anti-HBs/ad or anti-HBs/ay) were tested with human male and female leukocytes after having been absorbed with human female leukocytes. In Table 2 it is apparent that in no case was a significant level of rosette formation found with male leukocytes as compared either to female leukocytes or to male serum used as a negative control. Anti-HBs derived from female guinea pig was not available, and so could not be tested.

As a further test of possible specific binding of anti-HBs to male cells, each of the four anti-HBs sera (2 human and 2 guinea pig) was absorbed with human male or female leukocytes. The results in Table 3 show that no reduction in anti-HBs level occured as a consequence of absorption with leukocytes derived from either sex. Thus, potent and specific antisera to the a, d, and y antigenic determinants of HBsAg display no particular affinity for male as compared to female leukocytes.

Following these experiments, the sera from the human subjects whose leukocytes were used either for absorption or for rosette formation (17 in total) were tested for the presence of HBsAg and anti-HBs, and all were found to be negative.

Finally, three coded samples of mouse anti-H-Y sera (see Materials and Methods) were tested for reactivity with HBsAg using

erythrocytes sensitized with either HBsAg/adw or HBsAg/ayw. None of the three sera caused agglutination of either sensitized or control erythrocytes. The sera were also negative for HBsAg when tested by RIA.

A chance finding in experimental breeding of mice has relevance to the possible relations between infectious disease in the mother and sex ratio. The work was done independently of, and prior to, the present study. One of two control groups of splenectomized C57Bl/6 female mice injected with female spleen cells (same strain) was apparently infected with an unknown agent and had and unusual sex-ratio (males to females) which was significantly different from the other (uninfected) group, and from a third control group which was splenectomized only. [1]

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[1] All groups were part of a sex-ratio experiment in which animals were injected intraperitoneally 3 times with 2×10^6 spleen cells at 7 day intervals. The group of mice in question showed clear signs of disease after the second injection: some animals died, and the survivors (23 of 30 originally) gradually lost weight and fur. On circumstantial evidence, it could only be inferred that the spleen cell donor animal was a carrier of an infectious agent. When the surviving animals were bred, only 11 conceived and gave birth to 76 newborns with a sex-ratio of 48/28=1.714, which was significantly different from the two other control groups (see above) with sex-ratios of 143/153=0.935 and 72/68=1.059 (χ^2 test, $p<0.05$ and $p<0.1$, respectively). The deviated sex-ratio of the group in question

is thus attributed to the disease.

Discussion

The aim of this investigation was to test the hypothesis that the well- characterized determinants of Hepatitis B surface antigen crossreact with the male-specific H-Y antigen. On the evidence presented here, HBsAg and H-Y antigen do not share any common antigenic reactivity. Antibody to HBsAg, whether of human male or female origin , or of male guinea pig origin, showed no capacity to bind specifically to male cells (rat or human) when tested under two separate experimental conditions. Furthermore, antibody to H-Y antigen was not reactive with purified HBsAg conjugated erythrocytes. Thus, our experimental findings do not support the hypothesis proposed by Drew *et al.* (1978, 1978a) that HBsAg crossreacts with a male-associated antigen.

Although our experiments have been carried out with antigen and antibody reactants representing only four of the five well-characterized antigenic determinants of HBsAg (i.e., the major *a* determinant, and the minor *d*, *y* and *w* determinants), these are the determinants most likely to be found among HBsAg carriers in the Greek population studied by Drew *et al.* (1978). Since the minor *r* determinant is almost exclusively associated with carriers living in the Far East (Bancroft *et al.*, 1972· Mazzur *et al.*, 1973· LeBouvier *et al.*, 1973)) it is rare in Greece, and thus probably irrelevant in regard to a hypothetical crossreactivity with H-Y antigen as the cause of the altered sex-ratio observed in the offspring of Greek HBsAg carriers.

In our experiments we used anti-HBs raised in male guinea pigs. To achieve a final conclusion regarding crossreactivity between any one or more of the HBsAg determinants and H-Y antigen (or possible sex-related differences in the anitgenic composition of HBsAg generally) would require immunization of both male and female animals with HBsAg of each subtype derived from human HBsAg donors of each sex. It would perhaps also be necessary to take into account the antigenic variation manifested by the sub-categories of the major a determinant (Couroce-Pauty and Soulier, 1947), other "new" determinants which may be virus-specific (LeBouvier and Williams, 1975), and the host-coded proteins which are complexed in trace amounts with the 20 nm HBsAg particle (Neurath et al., 1974; Burrell, 1975). There remains the possibility that one of the two other antigenically distinct Hepatitis B Virus markers - namely HBcAg (Almeida, 1971) and HBeAg (Magnius, 1975) - may share antigenic crossreactivity with H-Y antigen. This latter possibility was neither studied nor taken into consideration by Drew and colleagues.

Although Drew et al. presented three lines of evidence to support their crossreactivity hypothesis (Drew et al., 1978a), they conceded that it cannot fully explain the observations of deviated sex-ratio in the Greek population. The hypothesis is also not supported by any evidence that males suffer from autoimmune conditions more often than females as a result of hepatitis B infection. Furthermore, it has been established that the H-Y antigen is the testis organizing agent of the mammalian indifferent gonad (Zenzes et al., 1978; Ohno, 1979; Wachtel and Ohno, 1979). Therefore, transmission of maternal anti-HBs, assuming crossreactivity with H-Y antigen, should interrupt the

function of H-Y antigen by competitive binding. This would eventually lead to hermaphroditism among male offspring of anti-HBs positive mothers. No unusual disturbance in sex determination has ever been recorded among populations in which Hepatitis B Virus is highly endemic. The fact that males are found to be chronic carriers of the virus more often than females may be the result of a more suitable environment (hormonal for instance), or perhaps of a specific affinity of the Hepatitis B Virus DNA to integrate into the Y chromosome. In this respect, our accidental finding in mice suggests that other conditions of disease in females might be associated with a deviated sex ratio.

In regard to the influence of parental experience with Hepatitis B Virus on the sex-ratio of children as reported by Drew et al., it would be interesting and important to know what the serological status of each individual parent was at the time of the mother's pregnancy. While HBV is highly endemic in Greece, published age-prevalence data indicates that both HBsAg and anti-HBs may be acquired by horizontal transmission of virus in adult life (Hadziyannis, 1975). It is also well known that spouses of HBsAg carriers are at particular risk of acquiring Hepatitis B infection, which is most often sub-clinical and manifested only by sero-conversion (Szmuness et al., 1975). Therefore, it cannot be assumed that all parents in the study were either HBsAg or anti-HBs positive during the early, procreative years of married life, and thus only one of the parents (HBsAg positive) may have been responsible for the sex selection of the offspring. It would also be of interest to know the serological status of the offspring at the time of birth: the hypothesis of Drew et al. would predict a higher incidence of HBsAg positivity in live-born males than in live-born

females, the male being relatively more tolerant.

Summary

The hypothesis that Hepatitis B surface Antigen (HBsAg) is crossreactive with the male-specific H-Y antigen has been tested. In none of three separate experiments using selected anti-HBs sera, or in a fourth experiment using anti-H-Y sera, could any evidence for crossreactivity be detected. Our results are therefore not compatible with the idea that altered sex-ratios observed among offspring of parents infected with Hepatitis B virus are related to crossreactivity between HBsAg and a male-associated

Note in proof

Since submitting the manuscript, we have obtained additional data to fortify our previous conclusions. Two human female anti-HBs sera have been tested for reactivity with H-Y antigen (antibody titers of these sera by PHA were 1:256 against HBsAg/ad for both antisera, and 1:256 and 1:4096 against HBsAg/ay , respectively: both antisera contained anti-HBs/y and anti HBs/a as determined following absorption with HBsAg of either subtype, ad or ay). After absorption with female mouse lymhocytes, both antisera showed only background levlels of rosette formation with either male or female mouse lymphocytes. Furthermore, we also tested the possibility that HBeAg crossreacts with H-Y antigen. Anti H-Y sera from five individual female mice showed no evidence for reactivity against HBeAg-positive human serum

as tested by gel immunodiffusion.

We have now confirmed that the two human sera previously tested (see Tables 1 and 3) contain anti-HBs/d as well as anti-HBs/a.

In a recent study, Werner et al. (Immunogenetics 8, 561, 1979) also failed to find evidence for crossreactivity between HBsAg and H-Y antigen.

CHAPTER VII

THE DEVELOPMENT OF TOLERANCE TO MALE SKIN GRAFT IN PRIMIPAROUS MICE

Introduction

Rejection of intrastrain male skin grafts (MSG) has previously been used to study changes in immune response to H-Y antigen in parous and splenectomized mice (Breyere and Barrett, 1960; Coons and Goldberg, 1978; Johnson, 1978; Lappe and Schalk, 1971; Streilein and Wiesner, 1977; Upholff, 1974). Although some of the reports are not concurrent with others, (Jeekel, 1973; Johnson, 1978; Vener, Martinez and Good, 1961), the data seem to indicate that parity results in increased tolerance to MSG, whereas splenectomy can result in accelerated rejection of MSG (Breyere and Barrett, 1960; Coons and Goldberg, 1978; Prehn, 1960; Smith and Powell, 1977; Streilein and Wiesner, 1977).

The spleen seems to play a role not only for the rejection of H-Y incompatible skin grafts but also in the maternal immune response to the fetal H-Y antigen during pregnancy (Lappe and Schalk, 1971 and Chapter VIII). Anatomic changes in the spleen during pregnancy and effects of splenectomy on placental and fetal weight have also been reported (Maroni and deSousa, 1973; Beer, Billingham and Scott, 1975). In the present study, MSG survival has been used as a probe to study the development of tolerance to H-Y antigen in virgin and primiparous mice which previously have been splenectomized , injected with male spleen cells or received both treatments.

Material and Methods

C57Bl/6 female mice, aged 8-10 weeks were splenectomized under

nembutal anaesthesia. Two weeks later the groups (B,D, and E see below) received 4 i.p. injections of 2×10^6 male or female spleen lymphocytes in 0.1 ml of phosphate buffered saline. This treatment was repeated at 7 day intervals. Animals were bred after the last immunization and the dams grafted with 10-16 mm^2 of male tail skin according to the method of Baldwin , Cohen and Hrapchak (1973). Instead of a gypsum cast a Band-Aid plaster was used to bandage the animals. Rejection was scored macroscopically, when at least 30% of the graft area was necrotized.

The animals were divided into five experimental groups : (A) untreated, (B) immunized with male cells ($H\text{-}Y^+$), (C) splenectomized, (D) splenectomized and injected with female cells ($H\text{-}Y^-$), and (E) splenectomized and immunized with male cells. These experimental groups were repeated in three categories of animals : virgin females, primiparous females transplanted within 1-6 days post-partum (prim. 1-6) and primiparous transplanted 10-20 days post-partum (prim. 10-20).

Statistical significance was determined by the Mann-Whitney (Wilcoxon) test followed by the Median test. The Kruskal-Wallis test, an extension of the Mann-Whitney test for multiple groups was used when necessary. The Median test was applied to show not only that there was a difference in distribution, but also that this difference resulted in a higher proportion of the group above the median. The Median test was automatically converted to Fisher's exact probability test whenever the combined sample size was smaller than 30. To isolate the statistical effect of the 'permanent takes' statistical significance was determined twice: once including the "permanent

survival" values ($\geq$ 100 days) and once excluding these values. The results of both analyses were virtually identical.

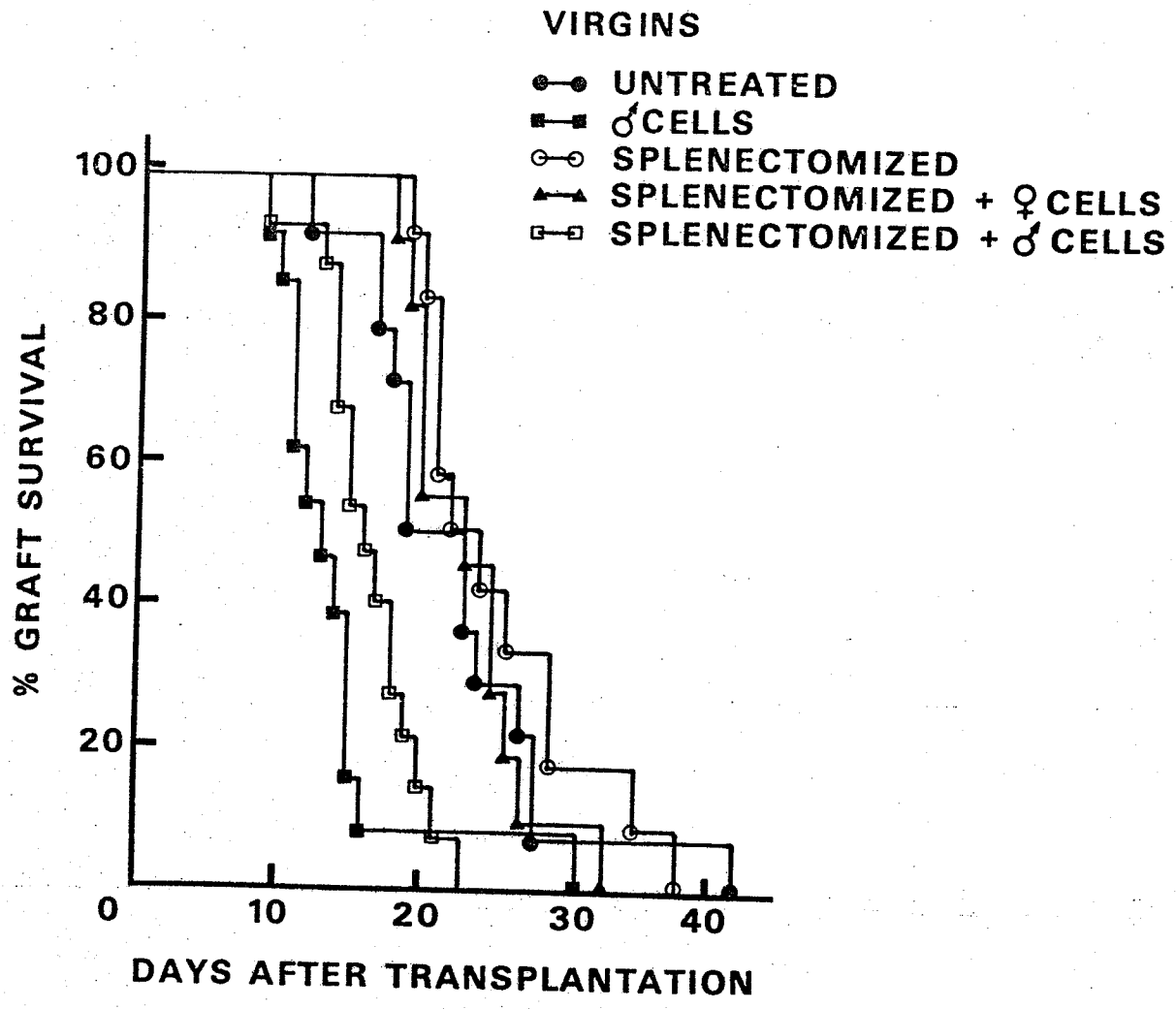
Results

Figures 1-3 present the cumulative % MSG survival for each treatment group in the three categories. The difference in the capacity of the various groups to reject MSG are particulary conspicuous at day 20 after transplantation. As can be seen (Figure 1), less than 15% of the virgin females which were stimulated with male cells (groups B and E) retained the graft at day 20 from transplantation, whereas over 50% of the females from the three remaining groups retained the graft at the same period. It can also be seen (Figure 1) that the curves for the H-Y stimulated females change closely together as is also the case for the other three groups. The picture is even more striking for the primiparous (1-6) category (Figure 2): here 90% of the females from group E and all the females from group B rejected the graft within 20 days, whereas only 65% of the splenectomized females and 45% of the untreated animals rejected the graft at 20 days. However, in contrast to the virgin females which rejected the graft in all cases, two animals (5%) from this category retained the graft permanently (>100 days). The picture for the prim. (10-20) category is quite similar (Figure 3): at day 20 after transplantation some 30-35% of the H-Y antigen stimulated animals (B,E groups) still retained the graft in contrast to 87-100% of the animals from the groups which were not stimulated to H-Y antigen. At day 30 from the surgery all animals from groups B and E rejected the skin graft and close to 50% of the other group still retained it (Figure

3). The overall survival of MSG for each category (excluding group D) is presented in Figure 4. It can be seen that at any given day a higher proportion of the primiparous (10-20) animals retained the graft and that the curves of the other two groups move close together . It is also noteworthy (Figure 4) that twice as many females in the prim. (10-20) category permanently retained the graft as those in the prim. (1-6) category. To facilitate the comparison of the treatment effect, the mean survival for each group and category was also summarized separately (Table 1). Besides the clear differences in the mean survival of MSG between groups which were sensitized against H-Y antigen and those which were not (see above) one can also note the sharp increase in the S.D. in the primiparous categories as compared to the virgin category. This is largely due to the higher proportion of 'permanent takes' among the parous females. The statistical analysis of these categories (Table 2) reveals that the distribution of MSG survival in the prim. (10-20) category was significantly different from the two other categories (virgin and prim. (1-6), which did not differ from each other. The effect of treatment within each category was further analysed by the Mann-Whitney and Median tests (Table 3). In all cases the survival of the MSG in the H-Y antigen stimulated groups was significantly shorter that in the untreated groups(A), the splenectomized group or (C) the groups which were splenectomized and injected with female cells (D). Therefore, the stimulation against H-Y antigen was effective in all cases irrespective of stage of parity, time of grafting (P.P.) or whether or not the animals were splenectomized. In none of the combinations were groups A, C, and D different from each other (Table 3). Thus, splenectomy or sensitization with female cells following splenectomy

Figure 1

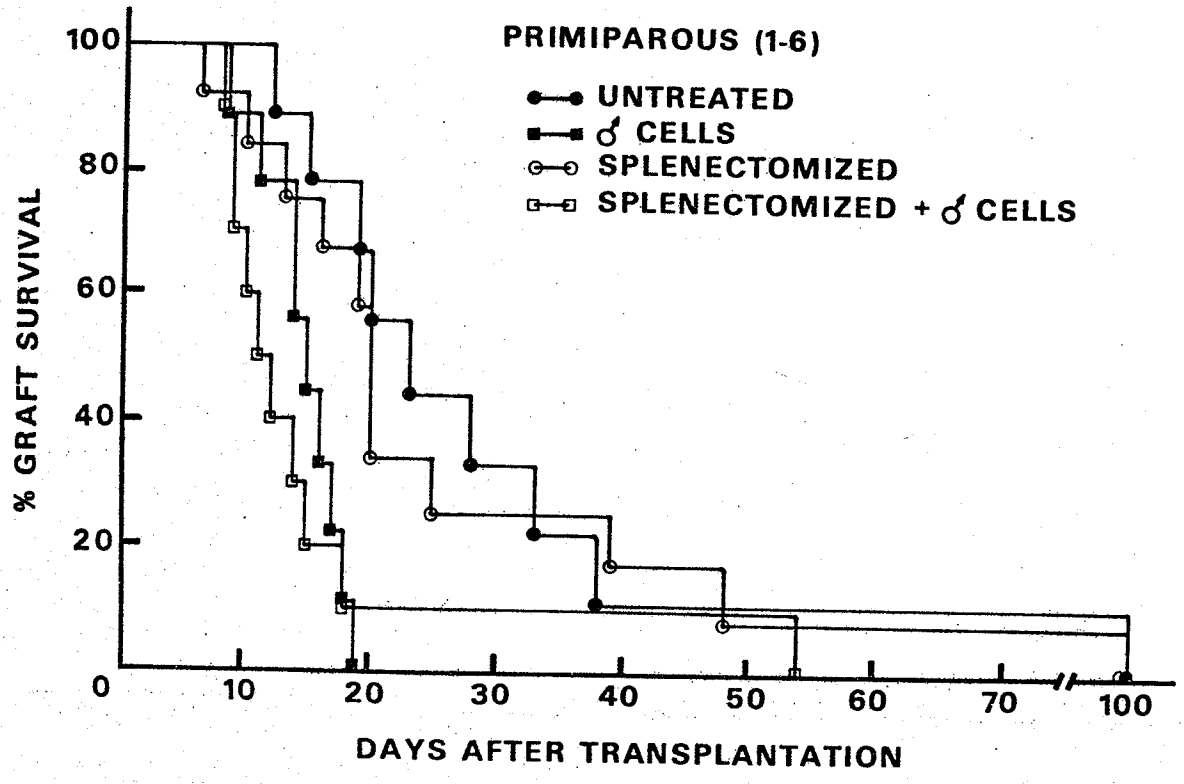
Survival of MSG in virgin C57Bl/6 females *



* Treatment groups are marked in the figure. For details see previous section.

Figure 2

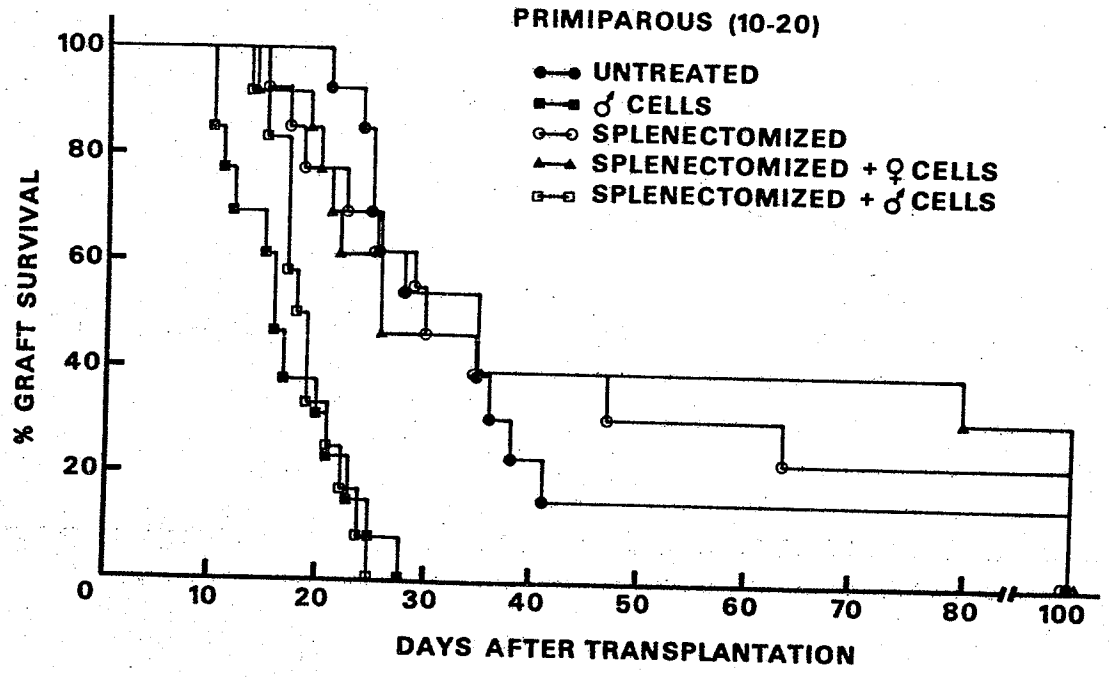
Survival of MSG in primiparous C57Bl/6 females which were transplanted within 6 days post-partum. *



* Treatment groups are marked in the figure. For details see previous section

Figure 3

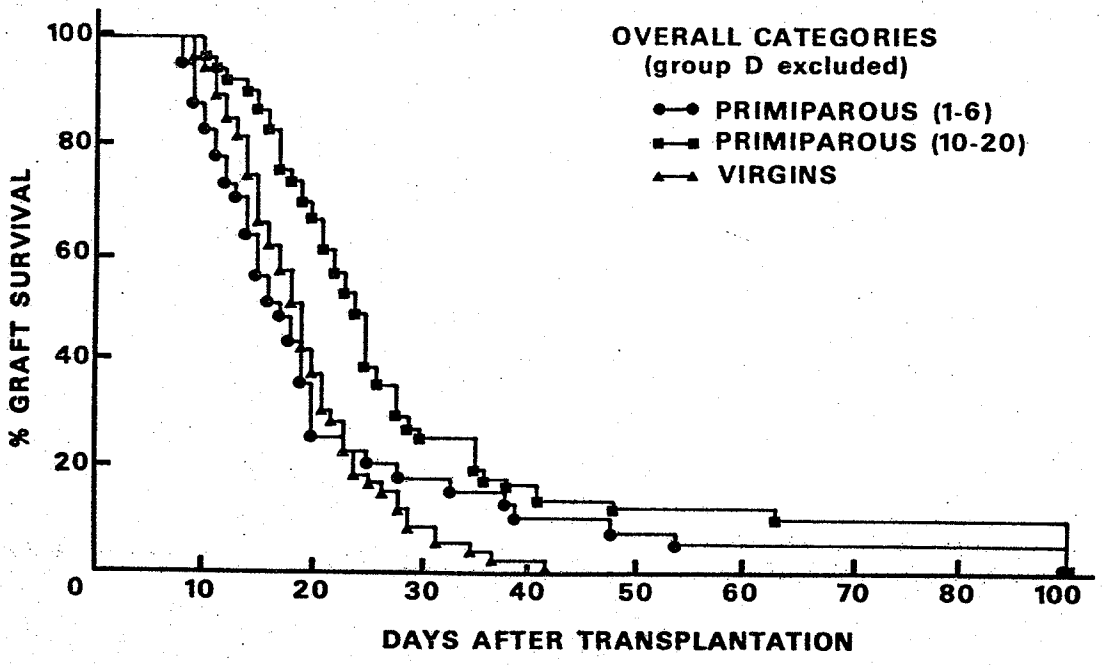
Survival of MSG in primiparous C57B1/6 females which were transplanted within 10-20 days post-partum. *



* Treatment groups are marked in the figure. For details see previous section.

Figure 4

Survival of MSG in the three experimental categories. *



* Treatment groups are marked in the figure. For details see previous section.

Table 1

The mean survival of male skin grafts on virgin and primiparous female mice

Treatment group*	Mean survival ± S.D. (days) for category		
	virgins	primiparous(1-6)	primiparous (10-20)
A	22.6±7.3	32.0±26.8	41.1±26.9
B	14.2±5.8	14.7± 3.5	17.2± 5.9
C	25.4±6.2	28.3±25.3	47.9±31.9
D	23.3±4.5		50.6±38.1
E	16.4±3.6	16.0± 3.7	19.0± 3.4
Total	20.1±6.9	23.0±20.7	35.4±28.7

*The treatment groups are described in the previous section.

Table 2

Comparison of male skin grafts survival among three experimental categories

Categories compared*	Statistical test			
	Median		Mann-Whitney	
	X^2	P	Z	P
prim. (1-6)/ virg.	0.52	NS	-0.89	NS
prim. (1-6)/prim.(10-20)	12.63	<0.01	-3.25	<0.01
Virg./prim. (10-20)	8.44	<0.01	3.50	<0.01

*NS = non-significant (P>0.05)

Since the category prim.(1-6) did not have the experimental group (D), this group was also excluded from the other groups when compared statistically. For category notations see Table 1.

Table 3

Analysis of pairs of treatment groups within three categories by the Mann-Whitney test *

Treatment groups compared	prim. (1-6)		prim. (10-20)		virg.	
	Z	P	Z	P	Z	P
A-B	2.52	<0.05	3.83	0.01	3.48	0.01
A-C	0.61	NS	-0.15	NS	-1.45	NS
A-D	-	-	0.41	NS	-0.80	NS
A-E	2.62	<0.01	3.93	<0.01	2.60	<0.01
B-C	-1.92	NS	-3.72	<0.01	-3.71	<0.01
B-D	-	-	-2.75	<0.01+	-3.57	<0.01
B-E	1.15	NS	-1.04	NS	-1.15	NS
C-D	-	-	0.52	NS	0.96	NS
C-E	2.15	<0.05	3.76	<0.01	3.84	<0.01
D-E	-	-	2.46	<0.05+	3.44	<0.01

*NS = non-significant (P>0.05)

For the notations of categories and treatment groups see Material and Methods. Results of the Median test for these data are not presented since they were practically identical.

+Not significant (P >0.1) after the exclusion of the 100 values.

did not affect the survival of MSG in virgin or primiparous females.

Discussion

Tolerance to MSG as a result of intrastrain mating is a well established phenomenon (Breyere and Barrett, 1960: 1960a: Prehn, 1960: Smith and Powell, 1977). Nevertheless , the mechanisms for the induction of tolerance to MSG through pregnancy are poorly understood. Recently, Smith and Powell (1977) suggested that thymus dependent (T) cells and not B lymphocytes or serum factors are responsible for the immune tolerance to H-Y incompatible skin graft in parous mice. In this respect to the development , the present data indicates that a single pregnancy was sufficient to induce complete tolerance ($\geq$ 100 days survival) to MSG in 14% of the primiparous mice which were transplanted at 10-20 days P.P. (Figure 4). Furthermore, while 67.3% of the mice from this category retained the graft for over 21 days, only 25% from the primiparous mice which were transplanted within 6 days P.P. and 40% of the virgin mice retained the graft for the same period.

These results suggest that the cytotoxicity to MSG was either augmented or not affected during the first 6 days P.P., and that tolerogenic (suppressor) factors to MSG became dominant at days 10-20 P.P.. This tolerance is clearly reflected in the data (Figure 4 and Tables 1 and 2). The tolerance to H-Y antigen (MSG) in the primiparous females (not injected with male cells) was provided by the male fetuses during pregnancy . From other studies (see Chapters VIII and IX) it was established that pregnancy can induce cytotoxic responses against H-Y antigen as well as production of H-Y antibodies.

Therefore, it must be assumed that the fetal H-Y antigen stimulated both immunity and tolerance (suppression) to H-Y antigen. The idea that immunity and tolerance can co-exist in the animal and are only two complementary sides of the same immune response was expressed long ago (Mitchison, 1964). This concept may be of special significance for the understanding of the fetal-maternal immune interactions. In vitro demonstrations of antibodies or cytotoxic cells against paternal antigens in parous mammals are apparently not correlated with any specific damage to the fetus .(This, however, does not exclude other affect on the conceptus.) On the other hand, there is much evidence for the presence of specific and non specific immunosuppressive factors during pregnancy (see Bernard, 1977; Clarke et al.,1978; Gudson, 1976; Hellstrom and Hellstrom, 1974). A self protective mechanism in which fetal alloantigens provoke an immune response in the mother that is balanced enough to outweight much of its own adverse effects is appealing and concurs with the current data on the maternal immune response to the fetal H-Y antigen.

The immunoregulatory role of the spleen and its being a source of suppressor T cells have previously been established (Amsbaugh et al., 1978; Anaclerio et al., 1979; Papiernia, 1976; Rozing et al., 1978; Skamene, 1977; Stolfi et al., 1977 and Ward and Munro, 1979). The possible role of the spleen in regulating the response to H-Y antigen (MSG survival) and sex ratio were also suggested (Coons and Goldberg, 1978; Streilein and Weisner, 1977; Lappe and Schalk, 1971). It was therefore of interest to examine the immuneregulatory role of the spleen in relation of tolerance due to parity. The present results showed no interaction between the survival of MSG and previous splenectomy in either virgin or primiparous mice.These results are in

accord with those of Johnson, 1978, who also found no effect of splenectomy on the survival of MSG. Thus, it remains open to question whether the spleen plays any role in the regulation of response to MSG.

Summary

The significance of splenectomy and prior sensitization against H-Y antigen on the response of primiparous C57Bl/6 mice to male tail skin graft (MSG) was tested. Splenectomy did not affect the survival of MSG in both virgin and primiparous mice. Primiparous mice transplanted within 6 days post partum rejected MSG at a similar pace as virgin mice; however, primiparous mice which were transplanted within 10 to 20 days post partum apparently developed tolerance to MSG. It is postulated that the male fetuses stimulate maternal cytotoxic and suppressor factors to H-Y antigen, and that an immune balance toward fetal alloantigens may be a mechanism to prevent adverse affect of the maternal immune system against the fetus.

CHAPTER VIII

PREGNANCY INDUCED H-Y ANTIBODIES AND THEIR TRANSMISSION TO THE FETUS IN RATS *

* In press in Immunology. By A. Shalev.

Introduction

Both humoral and cell mediated immunity to paternal histocompatibility (H) antigens during or following pregnancy, as a result of the stimulation provided by the fetus, have been reported in a variety of species using different methods (Feldman, 1972· Hellstrom and Hellstrom, 1974: Bernard, 1977: Carlson and Wegmann 1978: Baines et al., 1976· and Bernstable and Bodmer, 1978).

The H-Y antigen constitutes a special and interesting entity in this respect (Beer and Billingham, 1977) for several reasons: first, a crossreactive H-Y antigen has been identified in a great variety of vertebrate species (Wachtel et al., 1975: Shalev et al., 1978). Secondly, the H-Y antigen has now been recognized as the agent responsible for testicular organogenesis in mammals (Ohno et al.,1978; Zenzes et al, 1978, 1978a). As the only male specific H antigen known to exist, the maternal immune responses to H-Y antigen might affect sex ratio, fetal development and gonadal differentiation. The present study was conducted to examine antibody response of inbred DA rats to the fetal H-Y antigen as a result of a single or multiple pregnancy.

Material and Methods

Inbred DA rats aged 3-5 months were obtained and bred locally (Central Animal Care, Health Sciences Centre, University of Manitoba). The blood from the parous females, their offspring and control animals was collected separately and spun for 20 min. at 400 g. and 4°C.

The protein A rosette technique was selected for this study since staphylococcal protein A molecules bind specifically to the Fc portion of the IgG class of antibodies (Kronvall et al., 1970), the major class of antibodies which cross the placenta and is transmitted to the fetus (Brambell, 1970). A modification of the technique of Koo and Goldberg (1978) was used (see Part 1 and Chapter V). Negative controls (no serum) were run with each test and regularly showed less than 5% rosette formation. Results in which the difference in % rosettes between the tested sera samples and the negative controls were lower than 5% were disqualified.

Each serum was tested several times for capacity to induce rosette formation with male and female lymphocytes from DA rats. The results are presented as % specific rosette formation with male cells using the formula : (% rosettes with male cells - % rosetted with female cells) x 100 / % rosettes with male cells. Presence of H-Y antibodies was considered significant only if the mean % specific rosettes with male cells exceeded 2.5 standard error from the mean.

Results

Ten (52.6%) out of the 19 parous animals examined had detectable levels of H-Y antibodies in their blood serum (Table 1). Of these, 4 rats were primiparous and 6 were multiparous (2 or 3 pregnancies). The number of male offspring the female bore prior to testing did not seem to be correlated to the presence of H-Y antibodies in her serum since antibodies were detected in females with 2, 3,or 4 male offspring but not in sera from some females with 6,7, or 10 male offspring (Table

Table 1 : Presence of H-Y antibodies in the sera from parous rats

Female #	Number of litters (male newborns)	Day of bleeding post partum	% specific rosettes with male cells*	Presence of H-Y antibodies
1	1(4)	11	12.3 ± 9.3	-
2	1(2)	5	34.1 ± 7.0	+
3	1(4)	51	5.1 ± 8.5	-
4	1(5)	27	40.1 ± 6 4	+
5	1(3)	1	9.0 ± 13.4	-
6	1(3)	5	38.5 ± 4.5	+
7	1(6)	1	33.6 ± 5.9	+
8	2(10)	27	-4.4 ± 7.2	-
9	2(9)	20	21.3 ± 4.0	+
10	2(7)	19	30.7 ± 5.6	+
11	2(7)	33	0.0 ± 7.6	-
12	2(5)	3	30.4 ± 5.4	+
13	2(7)	39	1.8 ± 9.8	-
14	2(6)	12	1.8 ± 7.5	-
15	2(6)	14	30.6 ± 3.7	+
16	2(5)	2	3.8 ± 8.9	-
17	3(9)	1	35.6 ± 12.0	+
18	3(8)	20	24.0 ± 3.7	+
19	3(10)	11	7.7 ± 5.8	-

* Each figure represent the mean ± S.E. from 4-5 tests, calculated as described in the previous section.

Table 2 : The transfer of maternal H-Y antibodies to the offspring

Mother #*	Age of litter (days)	Male offspring: % specific rosettes with male cells**	Male offspring: Presence of H-Y antibodies	Female offspring: % specific rosettes with male cells**	Female offspring: Presence of H-Y antibodies	Female offspring: Presence of maternal H-Y antibodies
2	5	7.4 ± 13.2	-	43.0 ± 5.9	+	+
4	27	8.8 ± 5.4	-	17.5 ± 6.4	+	+
5	1	19.9 ± 13.7	-	21.6 ± 16.0	-	-
6	5	2.2 ± 7.5	-	36.7 ± 2.4	+	+
7	1	16.1 ± 17.0	-	24.6 ± 6.5	+	+
9	4	4.3 ± 9.0	-	13.7 ± 10.3	-	+
12	3	1.7 ± 14.7	-	47.4 ± 7.3	+	+
13	30	-	-	8.5 ± 8.2	-	-
14	12	-4.1 ± 8.4	-	1.3 ± 11.9	-	-
15	14	-0.6 ± 16.7	-	12.0 ± 15.4	-	+
17	1	2.8 ± 4.2	-	47.4 ± 10.7	+	+

* Numbers corresponde to Table 1

** Each figure represent the mean ± S.E. from 4 tests, calculated as described in the previous section.

1). Presence of H-Y antibodies in the sera from the parous rats was detected as late as 20 and 27 days post-partum as well as in the serum from a 27 day old female offspring (Tables 1 and 2). H-Y antibodies were detected in the sera derived from female-offspring in 6/11 (54.5%) litters but in none of the sera from 10 male litter-mates (Table 2). As shown (Table 2) , the presence of H-Y antibodies in the sera from the mothers was correlated with the presence of the antibodies in the female offspring, with the exception of two cases in which no antibodies were detected in the female-offspring despite the fact that they were present in the mother.

In addition, control sera from 6 DA rats (normal males and nulliparous females ,3-5 month old) were tested in the same way. The mean % male specific rosettes of 5 tests on each of these sera did not exceed 2.0 standard errors from the mean and therefore were considered non significant (random) results and H-Y antibody negative.

It is of interest to note that none of the sera that were tested gave a significant (>2.5 S.E.) negative mean value. Such a value would have indicated the presence of autoantibodies to a female-specific antigen .

Discussion

The significance of maternal immune response to alloantigens carried by the fetus has been discussed previously. According to one theory anti-fetal alloantibodies may serve as protecting (blocking) antibodies against the adverse effect of maternal cytotoxic cells (Hellstrom & Hellstrom, 1974; Bernard, 1977). Another theory suggests

that maternal stimulation to alloantigens carried by the fetus will benefit the conceptus with a higher rate of implantation and more vigorous development (Billington, 1964: James, 1965: Beer and Billingham, 1974, 1975, 1975a: Lappe and Schalk, 1971). In accord with the latter theory, the maternal immune response to a Y-linked (H-Y) antigen during pregnancy has been implicated in the increased weight of male placentae or male fetuses (Kirby, 1970: Ounsted & Ounsted, 1970: Vernier, 1975) and in changes of sex ratio (Beer & Billingham, 1977: Lappe & Schalk, 1971). The possible involvement of a male specific antigen in the development of toxemia of pregnancy in women has been proposed by Toivanen & Hirvonen (1970) based on their observations of a higher incidence of toxemia in pregnancies with a male fetus. Furthermore, these authors found an increase in sex ratio with the increased severity of toxemia. Borland et al. (1970) demonstrated a significantly reduced survival of male trophoblast cells (as compared to female cells) upon their transplantation into parous guinea-pigs, suggesting the specific immune destruction of male embryos. More evidence on the active immune response of pregnant females to the fetal H-Y antigen has been provided by Upholff (1977) who showed that lethally irradiated male mice which received maternal bone marrow suffered a reduced survival rate. The finding of Krco & Goldberg (1976) that 8 cell mouse embryos already express H-Y antigen is indeed consistent with the premise of in utero sex specific immune selection.

In a recent study Krupen-Brown & Wachtel (1979) reported presence of cytotoxic and agglutinating H-Y antibodies in the serum of multiparous mice from different strains. These authors were unable to correlate H-Y antibody synthesis with the capacity to reject male skin

grafts. In the present study, evidence is presented suggesting that the stimulus provided by the male fetus during a single pregnancy is sufficient to induce maternal production of H-Y antibodies. Moreover, within the limits of the size of the population studied, no evidence was found to support a possible correlation between the stage of parity (one,two, or three pregnancies) or the number of male offspring born, with H-Y antibody synthesis in the mother. This may imply specific suppression of antibody production in the animals which failed to respond. In relation to the suppression of the immune response to H-Y antigen , it is of interest to note a substantial body of evidence on the tolerance to H-Y antigen (male skin grafts) induced by parity in inbred strains (Billingham,1964; Porter & Breyere, 1964; Smith & Powell, 1977 and unpublished data). This tolerance seems to be mediated by T cells and not by B cells or serum factors (Smith & Powell, 1977). Nonetheless, the presence of immunosuppressive factors in the serum of parous females has also been widely established (Bernard, 1977; Clarke et al. 1978; Gudson, 1976 and Hellstrom & Hellstrom, 1974). It therefore appears that male conceptuses provoke a dichotomous (tolerance or suppression vs. cytotoxicity and antibody response) immune reaction in the mother. However, this presumes correlation between the in vitro and in vivo observations. This premise is further supported by a recent (unpublished) experiment in which I observed a significantly faster rejection of male skin grafts by primiparous C57Bl/6 mice which were transplanted within 6 days postpartum as compared to mice transplanted 4 to 14 days later. More specific studies on the nature of lymphoid cells triggered by the fetal H-Y antigen remain to be carried out in vitro .

In rats, prenatal transmission of maternal antibodies to the

fetus occurs through the yolk-sac. However, postnatal transmission is stronger and lasts for up to 20 days (Brambell, 1970). Since the half-life of immunoglobulin in newborn rats is 5.5 days (Solomon, 1971), the finding of H-Y antibodies in the sera from 1 day old as well as 27 days old female offspring indicates transmission of H-Y antibodies by both routes. The significance of H-Y antibody transmission to the fetus might lie with the specific ontogenic role of H-Y antigen (see introduction). It is possible that a high level of maternal H-Y antibodies can interfere with testicular organogenesis and possibly the development of male fetuses. Although such a situation is not likely to occur normally, it is possible in females with a history of intense exposure to male tissues (see Goulmy et al ,1978) or as a result of extensive cellular transmission from the male fetus(es) to the mother (see Beer & Billingham, 1976).

Summary

Rats of DA strain produce H-Y antibodies in response to the stimulation by their male fetuses. About 52% of the animals following a single or multiple pregnancy and 45.5% of litters (female offspring) had detectable levels of H-Y antibodies in their blood serum. Presence of H-Y antibodies did not appear to be correlated with the stage of parity or the number of male fetuses the female bore. H-Y antibodies were detected in the serum of a 27 days postpartum female and her 27 days old female offspring but not in any of the male offspring. It is suggested that H-Y antibodies are transmitted to the fetus in utero and to the neonates through the milk (colostrum). The possible significance of these findings in relation to the ontogenic role of H-Y antigen is discussed.

CHAPTER IX

EVIDENCE FOR THE ROLE OF THE MATERNAL IMMUNE SYSTEM IN BALANCING THE SEX RATIO IN MICE

Introduction

Factors affecting sex ratio have the subject of scientific interest for many years (Allan, 1959: Dahlberg, 1948: Weir, 1953: Kirby, 1970: Etzioni, 1968: Moran et al., 1969: Beatty, 1970). With the increased awareness of the significance of the immune system in fetal-maternal interactions (see Beer & Billingham, 1976), attention was given to the possible affect of such interactions on sex ratio. It was natural to link changes in sex ratio with an immune response to a male specific (Y linked) antigen. (For the sake of clarity reference to a male specific or Y-linked antigen will be made to H-Y antigen, the only male specific cell surface antigen which has been recognized to date). Salzman (1955) and Toivanen & Hirvonen (1970) suggested that their observations of more frequent toxemia of pregnancy in pregnancies with a male fetus and the increase in sex ratio with the increased severity of toxemia of pregnancy are interpretable in terms of potentiation of the maternal immune response to H-Y antigen. Kirby et al.(1967) proposed an explanation to the higher sex ratio among inbred human populations based on increased implantations of male embryos. Drew et al. (1978,1978a) strongly implicated crossreactivity between hepatitis B surface antigen (HBsAg) and H-Y antigen as an interpretation for their observations of outstanding changes in sex ratio in a highly infected Greek population and for the differential survival rates of kidney transplants from male donors in HBsAg and anti-HBs positive recipients. However,attempts to confirm this premise

have not been succesful (Shalev & Hannan, in press: Werner et al., 1979). Kirby (1970) proposed that H-Y antigen may interact with ABO blood group antigens and in this way influence sex ratio. The author presented data which suggested increased implantation of male embryos when the conceptus was ABO incompatible to the mother. Recent findings showing expression of H-Y antigen by 8 cell mouse embryos (Krco & Goldberg, 1976) and presence of H-Y antigen on mammalian erythrocytes (Shalev et al., 1978) furnish support to Kirby's hypothesis.

The only experimental evidence for the direct involvement of H-Y antigen in determination of the secondary sex ratio (presumambly through embryonic implantation) comes from the study of Lappe & Schalk (1971). In the present study an experiment has been undertaken to verify the effect of maternal immunization to H-Y antigen, with or without previous splenectomy , on the secondary sex ratio in C57Bl/6 mice.

Theoretical consideration

Assuming that the frequency of X and Y bearing spermatozoa is stable and that no sex-linked lethal genes occured, the secondary sex ratio (at birth) must be determined by maternal factors. Three principalt stages for sex-selection might be considered: selection prior to fertilization against X or Y bearing spermatozoa, selection against embryos at or shortly after implantation and selection at fetal stages. Among these the last is most simple to detect since it will result in high abortion rate and reduced litter size. Antigenic expression during the haploid phase of sperm production has never been

conclusively demonstrated in regard to X or Y linked genes. Therefore , there is no basis to believe at present that X and Y bearing sperm can be separated by immunological reactions. However, Bennet & Boyse (1973) claimed to have obtained a significant change in sex ratio in mice following treatment of the sperm with anti H-Y sera. This finding has not been confirmed and attempts to detect differential expression of H-Y antigen in spermatozoa have failed (Ohno & Wachtel, 1978: Ohno, 1979).

Implantation which is the establishment of firm cellular contact between maternal and embryonic tissues, is a fateful and vulnerable stage for the early embryo. Substantial evidence suggests that major embryonic mortality occurs during this stage (Hertig et al., 1957; Brambel, 1948; Adams, 1955; Wakasugi et al., 1967). Maternal immune preconditioning to paternal antigens which are expressed by the early embryo (Taylor & Hancock, 1975; Wudl & Chapman, 1976; Edidin, 1972; Johnson, 1976; Billington, 1976; Krco & Goldberg, 1975) may determine whether the embryo will vigorously implant and develop or whether it will be exposed to cytotoxic immune effects (Prehn & Lappe, 1971). Independently of maternal effects, the stage of implantation is also marked by rapid metabolic changes which may further increase its vulnerability to external factors (see Wakasugi et al., 1967).

The phenomenon of lymphocyte accumulation at implantation sites prior to and throughout implantation has been well established (see Johnson, 1976). These lymphocytes may well be the mediators of a specific immune reaction to paternal histocompatibility antigens during gestation. Indeed, Kirby (1970) presented data that anti lymphocytic serum can block implantation in the mouse. The specificity

and mode of action of the antiserum are not yet clear. Evidence that maternal response to paternal histocompatibility antigens is involved in determining the implantation potential of the embryos also comes from studies by Fekete (1947), Michie & Anderson (1966), Wakasugi et al. (1967): Palm (1970, 1974): Lappe & Schalk (1971): and Finkel & Lilly (1973). Clake & Hetherington (1971) tested the role of maternal preimmunization to paternal antigens on the formation of blastocyst induced decidual tissue. They found a significant reduction in the amount of decidual tissue in specifically immunized females but not in females which were sensitized to xenogenic antigens.

Based on the above discussion it appears that implantation is the most likely stage for immunological sex-specific selection to occur. Therefore, a theoretical model has been drawn which attempts to predict the consequences , in terms of sex ratio, of preconditioning to H-Y antigen in C57Bl/6 mice. The first assumption, based on the work of Vickers (1967), is that the primary sex ratio in the mouse is 1:1. Secondly ,based on the studies of Lappe & Schalk (1971) and Wakasugi et al. (1967) the figure of up to 30% difference between the number of potential embryos (corporea lutea) and the number of successful implants (fetuses) is adopted. Assuming a group of 10 females with 100 fertilized eggs, it is expected that at least 70 fetuses will develop successfully and will be born. Within the limits of the same litter size (7 per female), if full implantation of male fetuses took place the secondary sex ratio (males/females at birth) can reach 50/20=2.5 and the opposite figure (20/50=0.4) if full implantation of female embryos occured. Even more extreme sex ratios may occur if more than 30% of the fertilized eggs are eliminated. This however,should be reflected in the reduction of litter size. Following

this line of reasoning , the theoretical consequences of maternal immunization to H-Y antigen in terms of sex ratio and litter size are summarized in Table 1.

Material and Methods

Two separate experiments were conducted using 5-6 week old C57B1/6 mice. Animals were maintained in the same room under standard conditions and bred at the age of 3-4 months by caging 3 females with a male. Males were rotated among the cages after 15 days of breeding to avoid a paternal effect. Splenectomy was carried out under Nembutal anaesthesia; a small incision (5 mm.) was made on the left flank of the animal, the splenic blood vessels ligated, the spleen excised and the incision sutured. The animals were allowed to recover for two weeks before being immunized.

Experiment I - female mice were divided into five experimental groups: 1-untreated: 2-injected with male cells: 3-splenectomized: 4-splenectomized and injected with female cells: 5-splenectomized and injected with male cells.

Females were injected 3 times with male or female splenocytes (2×10^6 cells in 0.1 ml. phosphate buffered saline, intraperitoneally) derived from C57B1/6 mice at 7 day intervals. Breeding followed immediately after the last injection and continued for 66 days. Females which were not pregnant (hand examination) on day 35 of breeding were restimulated with the same dose of cells (group 2,4,5) Pregnant mice were separated into single cages commencing day 17 of

Table 1

Determination of sex ratio at implantation*

Situation	Conditions for embryos at implantation **	Embryos wasted ♂	Embryos wasted ♀	Average litter size	Expected sex ratio (♂/♀)	Detection
1	Normal	≤15	≤15	7	1	No statistical correlation between litter size and sex ratio
2	Favorable for males	<15	≤15	7-10	1-1.43 (50/35)	Increased litter size. More males can be expected with larger litters.
3	As situation 2, but maintains normal litter size at the expense of less females.	<15	>15	7	>1,<2.5 (50/20)	Higher sex ratio is not correlated to litter size.
4	Unfavorable for males	>15	≤15	3.5-7	0-1	Reduced sex ratio may be correlated to smaller litter size.
5	As 4, but maintains normal litter size by more implantation of females (favorable for females).	>15	<15	7	<1,>0.4 (20/50)	Reduced sex ratio is not correlated to litter size.

*Assuming a population of 10 female B6 mice with a primary potential of 100 eggs available.

**The hypothetical situation can be maintained immunologically by different reactions against H-Y antigen.

breeding and inspected daily for delivery. Newborns were sexed within 24 hours from delivery by identifying the pigmented spot at the base of the tail of male neonates (Lappe & Schalk, 1971 and confirmed by a chromosomes study of 20 randomely selected and coded male and female neonates). After delivery the animals were left in the same cage for another day to ensure that all neonates have been born (occasional delays of delivery are not uncommon in mice).

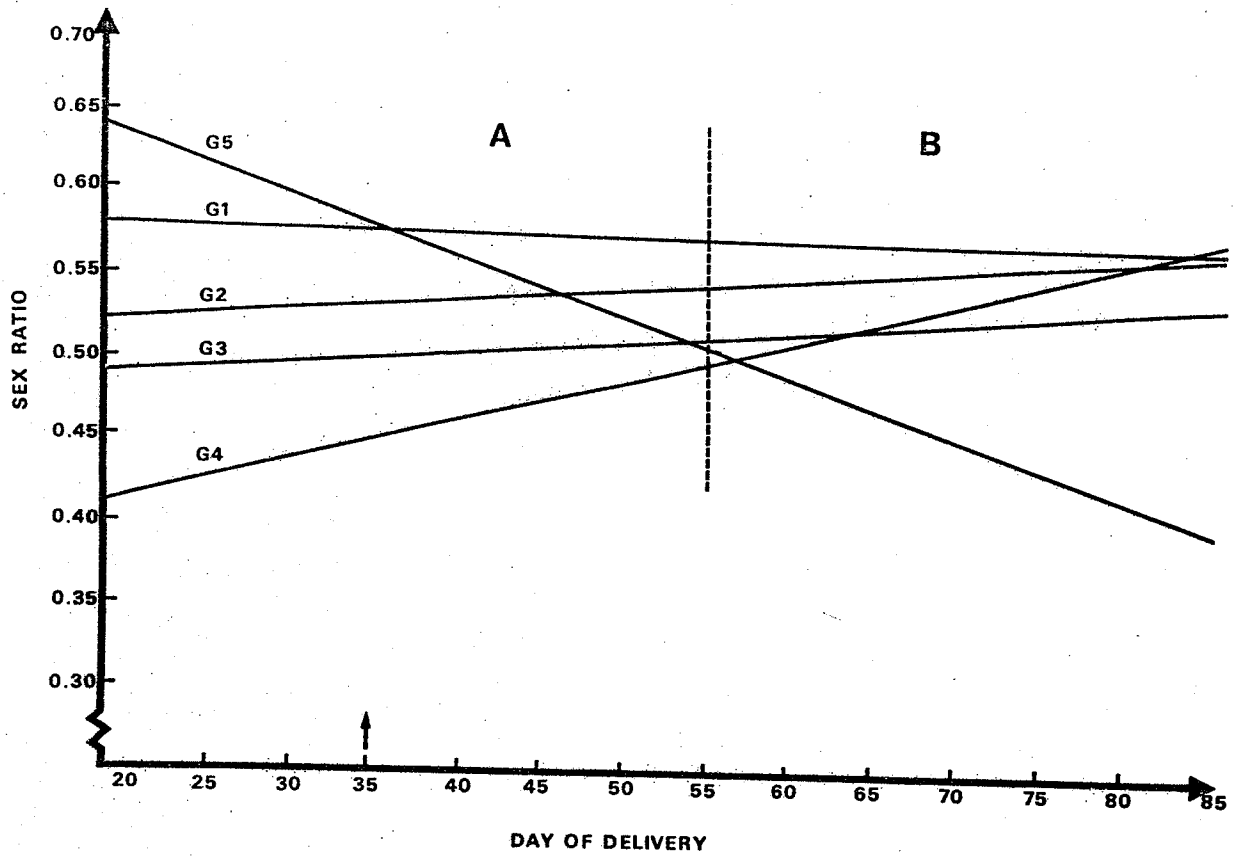
Experiment II -The same five experimental groups were used as in experiment I , but in addition two more groups were tested (see below). Other modifications from the first experiment include the immunization schedule ,the breeding period and the size of the groups, which was significantly larger. In this experiment females were immunized more intensively and for a shorter period of time; six injections of male or female splenocytes were given within two weeks at 2-3 day intervals (same dose and route as in the first experiment). Two additional groups were also restimulated with 2×10^7 male (group 7) or female (group 6) splenocytes 30 days after the last injection. All females in this experiment were bred for exactly 30 days in order to eliminate the possible effects of time (age variability in immune response).

Results

The results of sex ratio in experiment-I are presented in Figure 1 and Tables 2 and 3. The regression analysis (Figure 1) was carried out to detect skewing in the sex rate (males/litter size) as a function of the delivery time relative to the immunization treatment. As shown (Figure 1 and Table 2) only in group 5 (splenectomized and

Figure 1

Experiment I : Correlation between sex rate (males/litter size) and delivery day.



*The parameters if each Pearson's correlation are presented in Table 2. For obvious reasons the sex ratio values had to be converted to sex rate values. The arrow at day 35 marks the restimulation given to groups 2,4,and 5. Subgroups A and B are Marked by the broken line (see text and Table 3).

Table 2

The correlation parameters between the sex rate and delivery day (Experiment I) *

Group	n	b	r	F	P(F)
1	38	-0.0002	0.0366	0.048	0.827
2	43	0.0006	0.0885	0.323	0.573
3	41	0.0005	0.0811	0.258	0.614
4	19	0.0025	0.3420	2.250	0.152
5	39	-0.0036	0.4660	10.257	0.030

* The graphs are illustrated in Figure 1

Sex ratios in Experiment I*

Subgroup	Experimental group				
	1	2	3	4	5
	Untreated	Male cells injected	Splenectomized	Splenectomized + female cells injected	Splenectomized + male cells injected
A	(1A)	(2A)	(3A)	(4A)	(5A)
	1.113 (169)	1.073 (170)	0.949 (115)	0.818 (60)	1.310 (164)
B	(1B)	(2B)	(3B)	(4B)	(5B)
	1.239 (103)	1.051 (121)	0.926 (181)	1.353 (80)	0.719 (110)
Total	1.159 (272)	1.064 (291)	0.922 (196)	1.090 (140)	1.030 (274)

* Sex ratio=males/females . The number of offspring is given in parenthesis. Subgroups A and B represent the results from the conceptions that occured during the first 36 days (day 50 of breeding) and the results from the conceptions during the remaining 30 days (day 85 of breeding).

χ^2 (2 x 2) analysis of the above results : Sex ratios from the total values are not different from each other in all cases ($P>0.1$). When subgroups A and B were compared within each group, the difference was significant only for group 5 ($\chi^2=5.84$, $P<0.05$). Comparison of the same subgroup (A or B) between the groups indicated that the subgroup 5B was significantly different ($P<0.05$) from subgroups 1B ($\chi^2=3.89$) and subgroup 4B ($\chi^2=4.56$). The remaining comparisons were non significant at $\alpha=0.1$ level.

sensitized to H-Y) was the sex ratio correlated with the day of delivery (or day of conception). It is presumed that this significant correlation reflects a change in maternal immune response to the fetal H-Y antigen as a result of the active immunization to H-Y antigen. To test whether the change in sex ratio prior and after restimulation was significant, the sex ratio data for each group was divided into two subgroups according to the delivery day (Fig. 1) Group A = pooled sex ratio of deliveries during first 55 days of breeding (i.e. conception occured during days 1-36) and B= pooled sex ratio of deliveries during days 55-85 (i.e. conception occured during days 36-66). It is clear that animals of subgroup B (groups 2,4,5) received a restimulation injection prior to conception (several females might have conceived a short time earlier). The statistical analysis of these subgroups shows (Table 3) that a significant change in sex ratio was indicative only for subgroups 5A and 5B. However, it should be considered that because of this subdivision into smaller groups, the power of the statisitical test (χ^2) was significantly reduced.

As previously discussed, litter size may be an important indicator for understanding the mechanism of changes in sex ratio. The litter size of the groups was therefore recorded and analysed for possible correlation to sex ratio (sex rate). The mean $\pm$ S.D. of litter size in groups 1-5 respectively was 7.27 ± 2.05, 6.63 ± 1.83, 7.21 ± 1.96, 7.37 ± 1.83 and 7.00 ± 2.08. None of these values is different from the other when compared by a t-test ($P > 0.142$ in all cases). In none of the groups or subgroups was the sex rate correlated to litter size ($p > 0.232$ in all cases).

To conclude, the findings from the first experiment suggested the

following effects: a) Maternal splenectomy or sensitization to female cells following splenectomy did not affect sex ratio. b) Sensitization to H-Y antigen following maternal splenectomy may increase sex ratio if conception occured within 36 days after immunization. Later conceptions and/or restimulation to H-Y may have an opposite affect on sex ratio. c) No evidence for the effect of any of the treatments on litter size was evident, nor was litter size correlated to the sex rate of the litters .

Based on the results from the first experiment, the second experiment was designed to further verify these effects. It was predicted that an intense stimulation within a short period may enhance implantation of male embryos whereas a strong secondary response (restimulation) will result in a decrease in sex ratio. It was also reasoned that a maximal period of 30 days of breeding is essential to obtain a consistent effect. The results of sex ratios in the second experiment are presented in Table 4. No differences in sex ratio were found among the control groups (1,2,3,4,6) although a reduced sex ratio was indicative for the splenectomized group (3). Since a somewhat lower sex ratio was also observed in the first experiment (Table 3), the data from both experiments was pooled and compared to the combined data from the untreated groups; the difference was significant at $P \leq 0.025$ ($\chi^2=5.27$). The same analysis for the combined data for groups 2 and 4 was not significantly different from the untreated group ($P>0.3$). Sensitization to H-Y antigen following splenectomy resulted in a significant elevation in sex ratio. Thus, the sex ratio for group 5 was significantly higher than the sex ratio in any of the control groups (Table 4). This effect was particularly prominent when group 5 was compared to the splenectomized

Table 4

Experiment II. Sex ratio in the experimental groups and their statistical analysis.

Group #	Number of females	Treatment	Sex ratio (males/females)*
1	42	None	1.094 (312)
2	43	Injected male cells	1.085 (319)
3	57	Splenectomized (Spl.)	0.845 (404)
4	32	Spl. and injected female cells	1.009 (247)
5	72	Spl. and injected male cells	1.476 (520)
6	55	Spl. and restimulated with female cells	0.921 (415)
7	46	Spl. and restimulated with male cells	1.236 (315)

*Total number of neonates is given in parentheses.

Statistical analysis (χ^2 test, 2 x 2). Significant differences between the groups are as follows : 5:1; χ^2= 4.32, $P<0.05$. 5:2 ; χ^2= 4.63, $P<0.05$. 5:3; χ^2= 17.48, $P<0.001$. 5:4; χ^2=6.04, $P<0.02$. 5:6; χ^2=12.65, $P<0.001$. 7:3; χ^2=6.73, $P<0.01$. 7:6; χ^2= 4.08, $P<0.05$. The combined values from groups 5 and 7 were also signifcantly different from the combined values of group 4 and 6 (χ^2= 13.02, $P<0.001$) and from the splenectomized group (3) (χ^2=16.19, $P<0.001$).

group (3) (χ^2= 16.19, P<0.001). The restimulation 30 days after immunization to H-Y antigen and splenectomy (group 7) also resulted in a sex ratio which was significantly higher than that of the splenectomized group (3) and the equivalent female-cells-restimulated group (6) (Table 4), but was lower than the sex ratio of group 5. Although the drop in sex ratio caused by the restimulation is not statistically different from immunization alone (group 5) and is also not to the extent observed in group 5B (experiment I; Table 3), this result supports the suspicion originated from the finding in the first experiment about the effect of restimulation to H-Y antigen on the sex ratio.

As in the first experiment, litter size was recorded and analyzed for possible correlation to sex rate. The mean $\pm$ S.D. litter size for groups 1-7 respectively was : 6.93$\pm$1.80, 7.44$\pm$1.48, 7.02$\pm$1.80, 7.73$\pm$1.61, 7.29$\pm$1.67, 7.42$\pm$1.92, 7.65$\pm$1.72. None of these values was found statistically different from the other (t-test, P between 0.1 and 0.9). The litter size was also not correlated to sex rate (P range 0.061-0.491) excluding group 3 (r=0.363, P=0.03).

In addition to the above parameters, in this experiment the breeding efficiency (#deliveries x 100 /# animals bred) and mean breeding+gestation period were also recorded and analysed statistically. Breeding efficiency ranged between 75.4% and 88.7% and none of the groups was different from the other in this respect (P>0.05, χ^2 test); the mean breeding+gestation period $\pm$ S.D. for the various groups ranged from 29.26$\pm$9.39 to 33.90$\pm$10.78 and none of the groups was different from the other (P>0.05) in this respect (Mann-Whitney test).

Discussion

The evolutionarily highly conserved H-Y antigen, like other highly conserved molecules (Ogievetskaya, 1979) does not evoke a strong immune response upon immunization of inbred females with male cells. Furthermore, the possible presence in fetal ovaries of a diffusible factor which can block the binding of H-Y antigen to ovarian cells (Wachtel & Hall, 1979) may suggest that females can recognize part of the H-Y antigen complex as a 'self' molecule. Therefore, immunization to H-Y antigen was not expected to effect the survival or implantation of male fetuses to any extreme extent. Unlike the situation in rats, in which maternal sensitization to paternal antigen can result in runt disease and fetal destruction when particular crosses are made (Palm, 1970, 1974; Milgrom et al. 1977; Beer & Billingham 1973, 1976),sensitization to H-Y antigen through the sperm, fetal contact, cellular transmission or active immunization normally does not cause measurable changes in sex ratio (Lappe & Schalk, 1971; McLaren, 1962). It would have been very surprising ,indeed, if sex ratio was normally vulnerable to the immune status of females relative to H-Y antigen. Such a situation would have allowed strong and negative selective pressures. It was already pointed out that natural selection for factors which maintain sex ratio balance are extremely strong (Hamilton, 1967; Leigh, 1970). However, the question remains : is the sex ratio maintained only due to random fertilization by X or Y bearing spermatozoa?. Evidence that this may not be the case was suggested by Verley et al. (1967) who showed that the variability of sex ratio in individual mouse litters was less than

expected by chance alone. Do immune factors such as blocking (enhancing) antibodies which have been widely demonstrated in mammalian pregnancies (Bernard, 1977) or specialized lymphoid cells, such as suppressor T cells, play a role in balancing the natural sex ratio ?. The evidence that the maternal immune system is stimulated and responds to the fetal H-Y antigen is impeccable (Smith and Powell, 1977: Streilein and Weisner, 1977: Upholff, 1977: Krupen-Brown and Wachtel, 1979: Shalev, 1980), yet there are no data to show that this response may be related to sex ratio. If the maternal immune system plays a role in maintaining the normal sex ratio, it has to be a well balanced role which prevents extreme changes in the secondary sex ratio. Nonetheless, if this natural immune balance can be disturbed by removing a central lymphoid organ (such as the spleen) which is responsible for maintaining the immune balance, changes in sex ratio might be observed. The results of this study show that maternal splenectomy alone significantly reduced the sex ratio, but when active immunization to H-Y preceded splenectomy, the sex ratio was significantly elevated. Lappe & Schalk (1971) originally discussed the possible role of the spleen in maintaining the normal sex ratio. It was suggested that a spleen factor has a blocking effect on the implantation of male conceptuses , which otherwise are at advantage in implantation due to their endowment with H-Y antigen. This theory was put foreward in contrast to another theory (see same paper) which suggested that the spleen excretes factors (antibodies) to protect the fetus from the harm of maternal cytotoxic cells. A difficulty in Lappe & Schalk's theory is that , if the spleen has a preventive effect on implantation of male embryos, a higher implantation rate of male conceptuses is expected in splenectomized females. The results

presented by the above authors (Lappe & Schalk, 1971;note in proof) showed no change in sex ratio of splenectomized females. Furthermore, the results in the present study are even contradictory and indicate that splenectomy alone reduced the sex ratio. The latter finding alone would rather suggest that the spleen has a protective role on the implantation of male embryos. In view of the data obtained in this study, the knowledge accumulated in recent years on the immunoregulatory role of the spleen and the high complexity of the fetal-maternal immune interactions , both theories appear over simplified. Based on the present findings , the role of the spleen in maintaining the normal sex ratio may at best be described as that of a "buffering" regulator : in its absence sex ratio seems to be allowed to shift in either direction, depending on the treatment applied.

Considering that none of the treatments significantly affected the mean litter, size , nor was litter size correlated to the sex ratio in the litter the hypothetical situation which best matches the results for subgroups 5A (Exp.I) and groups 5 and 7 (Exp. II) is situation 3 in Table 1; while subgroups 5B and splenectomized groups best match situation 5 in Table 1. The expected sex ratios range for situations 3 and 5 respectively (Table 1), are 1-2.5 and 0.4-1. Indeed, the values obtained in both experiments are within this range. The highest and lowest sex experimental values obtained were 1.476 and 0.719, which is within the expected ranges, but suggests that the capacity to intervene with the inherent sex ratio by the immunological procedure applied was rather limited. Noteworthy, the value 1.476 is very close (and probably undistinguished) from the figure 1.465 which was obtained by Lappe & Schalk (1971) when applying the same principal treatment to C57Bl mice.

Prehn & Lappe (1971) distinguished between three different patterns of maternal immunization to paternal antigens prior to conception. They suggested that vigorous immunization against strong histocompatibility (H) antigens will mildely jeopardize fetal survival, while immunization to weak paternal H antigens will increase the selective advantage of the conceptus. Lappe & Schalk (1971) also showed that the increased capacity to reject male skin grafts was associated with a higher sex ratio. Apart from the difficulty to define "strong" and "weak" immunizations, the results in the present experiment indicate that the relative timing of immunization and of conception are of importance in determining the secondary sex ratio. Other factors (such as dose and route of immunization) which were not examined during this study may also be significant. Within the limitations of the study it appeared that the restimulation with male cells 30 or 35 days after the primary immunization resulted in a decrease in sex ratio. This suggests that the restimulation to H-Y antigen evoked in the females an immune factor which balanced and even out-weighted (Exp.I) the immune factors which provided advantageous conditions for the implantation of male embryos.

Finally, the present study strongly supports a limited role for the maternal immune system in balancing the secondary sex ratio in mice. It is hoped that future studies will be able to indicate the maternal immune factors which are involved in maintaining and controlling the sex ratio in mammals.

Summary

Two experiments have been conducted to verify the effect of maternal preimmunization to H-Y antigen on the secondary sex ratio in C57Bl/6 mice. In accord with the theoretical model previously set, it was found that extensive immunization to H-Y antigen following splenectomy resulted in a significant increase in sex ratio (males/females) without affecting litter size. Litter size was also not correlated to sex ratio. The data from both experiments suggest that splenectomy alone or restimulation to H-Y antigen (after 30 days) both act to decrease sex ratio. It is concluded that the maternal immune system plays a limited, but significant ,role in maintaining the normal sex ratio in mice probably through balancing the immune conditions which provide advantageous implantation rate to male embryos.

PART 4

GENERAL DISCUSSION

Twenty five years ago two investigators published a short communication on a "peculiar" phenomenon: female mice rejected skin grafts from males within well established inbred strains (Eichwald and Silmser, 1955). For about another twenty years no one suspected the possible significance of their observation. The question of whether this male specific antigen (later established to be Y-linked and termed H-Y) was genetically constitutive or was inducible by male hormones was hotly debated. The breakthrough was fast after the development of an assay for *in vitro* detection of H-Y antigen. Once the evolutionary distribution and conservation of the antigen were demonstrated (Wachtel *et al.*, 1975), the idea that H-Y antigen could be the long-sought male-determining product was also expressed (Wachtel *et al.*, 1975b). Since then, this field of research achieved increasing momentum. Today , after 200 published studies the evidence leaves no doubt as to the major role of H-Y antigen in sex determination. To date, we are standing at the beginning of a new era in understanding and controlling the process of sex determination in mammals.

The research presented in this thesis was carried out during the period 1977-1980 and reflects some of the difficulties and the rapid changes which characterized the field during that period. Some of the inherent difficulties were methodological. As discussed earlier (Part 1 and Chapter I), the weak nature of H-Y antigen (and antibodies) seriously hampered the application of some useful immunological techniques such as radioimmunoassay, column binding, etc. One can now say that these type of difficulties will disappear in the foreseeable future, as H-Y antigen has been isolated and purified from human cells

(Nagai et al., 1979) and specific H-Y antibodies were produced by the hybridoma technique (Koo, personal communication). The classic transplantation methodology which is dependent on a specific (cell mediated) immune response to H-Y antigen is useful to measure in vivo changes in the response to H-Y antigen such as caused by splenectomy or pregnancy (Chapter VII). However, the transplantation methodology has disadvantages in respect to the natural biological variability among animals, the extended time of male graft survival and variability in transplantation techniques from one laboratory to the other which influence the results (Baldwin et al., 1973). Phenomena such as crossreactivity of the antigen or the expression of H-Y antigen on small cell samples could be resolved only after the application of the in vitro methods. Yet, even after several in vitro methods for H-Y antigen typing have been developed, the weak nature of the reactions (which also resulted in variability) remained a major problem (Shalev et al., 1978). The protein-A technique falls within this category. Although the method has the advantages of low background levels ($< 5\%$ rosettes without serum) and fair reproducibility, as in other methods the specific reactions with H-Y antisera are low in comparison to antisera of known strength (30-40% compared to 80-100% rosettes at the same dilution). Nonetheless, the PA rosette technique has special application because of the fact that it detects antibodies of the IgG class (Chapter VIII).

The evolutionary conservation is one of the outstanding features of H-Y antigen. Indeed, it is very understandable that molecules mediating organogenesis , and especially sex determination would show such conservation. In this respect, H-Y antigen may be different from other (hypothetical at present) organogenesis antigens in the sense

that sex (in a broad sense) is virtually common for all living organisms and that in principle only one sex can express a sex specific (H-Y) antigen. The possible significance of the evolutionary conservation of H-Y antigen and its relation to sex determination in non-mammalian classes have been discussed earlier (Chapters II,III). While the mysteries of sex determination in mammals will probably be unravelled in the foreseeable future, the understanding of the mechanisms of sex determination in other animal classes and their evolution would probably require greater efforts and much research. The reasons for this were discussed by Ohno (1979) who emphasized the principal difference in sex determination between mammals and non-mammals. What, if any, is the role of H-Y (or an H-Y crossreactive) antigen in the process of sex determination in non-mammalian animals ? In the same context ; why is H-Y antigen associated with the heterogametic sex rather than the same sex in various classes (e.g. Mammalia , Aves, Amphibia). If one is to accept that the H-Y structural gene(s) was preserved on a differential region of an ancestral sex chromosome (the hemizygous chromosome?), during 400-500 million years (invertebrates to mammals) then it must be assumed that H-Y antigen played an important ,or even crucial role in the evolution of sex determination. The fact that H-Y antigen in mammals was preserved on all somatic tissues, rather than testicular cells alone, despite lack of evidence that it fulfills any vital role besides testicular organogenesis, may further be used to argue that originally it could have been engaged in other functions besides testicular organogenesis. Another line of argument, which I consider less likely, is that H-Y antigen was conserved despite its being of little or no significance for sex determination in lower vertebrates

and invertebrates. If this is correct, one must still assume that at some early stage H-Y antigen has had a vital role, but that during the evolutionary metamorphoses of the mechanisms of sex determination this role could have become of marginal or no significance. The utilization of tight genetic control on sex determination in mammals could have re-established a major role for H-Y antigen. Expression and control on sexuality in E. coli can serve as a basic model to understand the evolvment of sexuality through sex specific antigens (Monroy and Rosati, 1979 and see Chapters III). The genetic essence of sexuality in E. coli is an extra-chromosomal piece of DNA (episome or plasmid) of about 45×10^6 daltons which comprises about 2% of the bacterial genome and carries about 100 genes (Willets and Broda, 1969). This factor, known as F-factor (F=fertility) , is responsible for the production of 1-4 male specific hollow tubes (F-pili) which are imperative for adhesiveness (conjugation) and transfer of DNA from the F^+ cells to the F^- cells (Briton, 1965). In addition the F factor codes for a male specific antigen (f^+) and is responsible for the absorption of male specific RNA bacteriophage (Orskov and Orskov, 1960: Briton, 1965). Considering the wide distribution of sex specific antigens of which many are engaged in cellular recognition (Chapter III), the investigation of the possible evolutionary association of these phenomena to the evolution of sexuality and self/non-self recognition might yield interesting findings.

A question which is unsettled as yet in relation to the genetic conservation of the sex determination mechanism in mammals is where does the H-Y locus (loci) reside in the genome. As discussed earlier (Chapter V), the present data allows more than one interpretation. In order to fully understand the genetics and evolution of sex

determination in mammals this question will have to be resolved. The two major options are that the H-Y structural locus (loci) in mammals is normally located on the Y chromosome , but may be translocated (relatively high incidence) onto the X chromosome or autosomes. Otherwise, H-Y loci reside on chromosomes other than the Y chromosome but are activated through a Y-linked gene(s). A third alternative is that the location of H-Y genes is not identical in various mammalian species, which is as valid as the two other options. Our study of the family of polled goats (Chapter V) confirmed the expression of H-Y antigen in functional females (see also Selden et al., 1978; de la Chapelle et al., 1978). These observations not only establish the quantitative significance of H-Y antigen as a testicular organizing agent, but also suggest that ovarian organogenesis can proceed normally despite the presence of minor quantities of H-Y antigen. If ovarian organogenesis were a "passive" event which occurs in the absence of H-Y antigen, it could be expected that the presence of even minor quantities of H-Y antigen would have a dominant effect towards testicular organogenesis (or at least intersexuality). Therefore, this may serve as indirect evidence that there might be another substance in female fetuses which competes with H-Y antigen and is responsible for ovarian organogenesis. Indeed, the existence of such substance has been predicted long ago (Hamerton, 1968) and recent experiments indicate its possible existence (Wachtel and Hall, 1979).

Various mutants of sex determination have been examined for H-Y antigen in recent years. These include the testicular feminization syndrome (Tfm); XX males and true hermaphrodites (human, dog), pseudohermaphrodite goats , XY gonadal dysgenesis, the sex reversed mouse (Sxr) and the bovine freemartin (the latter abnormality is not

genetic). The data from these studies contributed considerably to the understanding of their etiology and the mechanism of sex determination in mammals. Besides the scientific significance of pointing out the etiology of a given situation, other potential clinical applications of H-Y typing in respect to sex determination are still unclear. Unlike sex hormones which are effective at the adult stage of life, H-Y antigen is effective only during a short period of gonadal organogenesis when its presence is crucial. Therefore, later application will probably be useless in regard to the gonadal formation and function. A clinical aspect which may be of great potential in medical application is that of male to female organ (or tissue) transplantation. Two recent studies by Goulmy et al. (1977, 1978), have convincingly demonstrated the existence of a specific cytotoxic response to H-Y antigen in females who previously received transplants from compatible males donors. The detection of these responses is vital for the survival of such patients.

A major part of research in this thesis was devoted to investigate the possible role of H-Y antigen in fetal-maternal immune relationships. Observations such as selective mortality in utero, higher prevalence of certain diseases in males, changes in sex ratio and placental size have previously been attributed to the maternal immune response against the fetal H-Y antigen (see Chapters VI, VIII and IX). However, one should be cautious about these interpretations, since males and females can be selected on the basis of other inherent traits such as activation in an early female embryo of both X chromosomes, which results in an double dose of the X-linked gene products (Epstein, 1978), and genetic difference in growth rate

between male and female fetuses (Scott and Holson, 1977). Could such differences be responsible for a differential resistance to pathogens at early development?. Nevertheless, experiments involving histocompatibility (H) antigens (not H-Y) in mice and rats indicate that in principle, fetal alloantigens do play a role in determining embryonic implantation, fetal survival and fetal and placental size, by specific in utero sensitization of the maternal immune system (Part 1 and Chapters VII-IX). The H-Y antigen is not completely comparable to strong H antigens because of its relative weak immunogenicity, its ubiquitous distribution in nature and its major role in sex determination. These factors probably created during the evolution a selective pressure to eliminate the adversity of maternal immune responses against mammalian male conceptuses. As discussed earlier (Chapters VII-IX) the evidence for the maternal immune response to the fetal H-Y antigen is impeccable, however , there is no evidence as yet that these reponses can influence testicular organogenesis (by transmission of H-Y antibodies) or the survival and development of male fetuses. The biological sense for the lack of such effects were discussed earlier (Chapter IX).

The studies and discussions presented here on the role of H-Y antigen in fetal-maternal immune interactions (Part 3) illustrate that the contemporary knowledge on the subject is still limited and indirect. The H-Y system provides a unique set of experimental conditions which can be rewarding to the investigator. While in examining the role of other H antigens in fetal-maternal immune interactions the conclusions in regard to maternal effects are complicated by the genetic heterozygosity of the fetuses (unless syngeneic strains are used), this problem does not exist in regard to

H-Y antigen. Furthermore, the special significance of H-Y antigen in mammalian sex determination and its wide evolutionary distribution make it of particular interest from this point of view. Theoretically the understanding of the fetal-maternal immune relations in regard to H-Y antigen is a potential way to control the fetal sex, to control sex ratio and to predict the sex of the fetus (human) (detection of H-Y antibodies in the mother or another specific response to H-Y antigen). At present, the latter seems to be the most applicable use for H-Y antigen in this respect. The development of a simple test to detect an anti H-Y response in pregnant women may become a realistic application in the near future.

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