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The case of the midwife scientist

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ABSTRACT Genes controlling both testis determining and expression of the male-specific transplantation antigen, HY, are located on the short arm of the mouse Y chromosome, and on the X and Y-linked translocation, Sxr^a. A mutation of Sxr^a was discovered in a cross between an Sxr carrier male and a T16H/X female. This was designated Sxr^b and found to affect both the expression of HY and spermatogenesis, but not testis differentiation, thereby disproving Ohno's hypothesis that HY controlled testis determination. Molecular genetic analysis showed the mutation to be caused by fusion of two duplicated genes, *Zfy1* and *Zfy2,* deleting the intervening DNA. This deletion interval, ΔSxr^b, contained a number of genes, each a candidate HY gene. Expression cloning with HY-specific T cell clones identified Smcy, Uty and Dby as encoding peptide epitopes of this transplantation antigen. The human homologues SMCY and UTY likewise express HY antigens and these are targets of damaging graft-versus-host (GVH) responses and potentially therapeutic graft-versus-leukaemia (GVL) responses following bone marrow transplantation (BMT). Knowledge of the peptide identity of HY epitopes allows monitoring of immune responses following BMT, using fluorescent tetramers, and also offers the possibility of inducing immunological tolerance.

KEY WORDS: *Sex determination, spermatogenesis, transplantation, HY antigen, expression cloning.*

Brought together by translocation

There were two sorts of translocations that brought me together with Anne McLaren in the late 1970s. One was geographical and the other, Sxr, genetic. Anne had moved to London from Edinburgh to found the MRC Mammalian Development Unit, and a young Japanese postdoctoral fellow, Takeshi Matsunaga, was working there. He had previously moved from Japan to the Basle Institute for Immunology to do his PhD, and he was now focused on questions of self/non-self discrimination, aspects of which could be well addressed in tetraparental chimeras, the generation and analysis of which Anne had pioneered (McLaren, 1976). This was an area which also interested me, and Takeshi made contact about setting up collaborative experiments (Matsunaga & Simpson, 1978; Matsunaga *et al.*, 1980). Anne was however no stranger to immunology, which then as now permeates many areas of biological science. The conundrum set out by Peter Medawar, on 'the foetus as an allograft' was on the borders of her territory in mammalian development *in utero*, and the notion of cell-mediated immunity of the sort manifest by graft rejection she found interesting, albeit rather daunting in its apparent complexity. Anne's ability to unravel the genetics of sex determination was a subject of my admiration.

Ohno in the mid-1970s had been intrigued by the discovery of 'MHC restriction' (Zinkernagel & Doherty, 1974), in which the T lymphocytes that effected cellular immune responses appeared to recognise foreign molecules only in the context of their own specialised cell surface glycoprotein molecules. These were encoded by genes within the major histocompatibility complex, MHC (H2 in mice, HLA in humans). Ohno proposed that recognition of the male-specific transplantation antigen, HY, which I had shown to be recognised by cytotoxic T lymphocytes in an MHC restricted manner (Gordon *et al.*, 1975), but against which antibodies had also been reported (Goldberg *et al.*, 1972), was the trigger for male differentiation during embryogenesis (Ohno, 1977). Data consistent with this hypothesis had been reported (Bennett *et al.*, 1977) using X/XSxr sex-reversed male mice, which tested positive with antibodies against 'HY'. Since at that time I had some doubts about whether the HY molecules recognised by antibodies and T lymphocytes were the same, we set out to ask whether X/XSxr male mice also possessed the HY antigen recognised by cytotoxic T cells. These experiments were carried out in collaboration both

Abbreviations used in this paper: BMT, bone marrow transplantation; GVH, graft virus host; GVL, graft versus leukaemia; H, histocompatibility; HY, male-specific transplantation antigen; HVG, host versus graft; MHC, major histocompatibility complex; SDM, serologically defined male; Sxr^a, an X and Y-linked translocation

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with Steve Wachtel, who supplied X/Y and X/XSxr male mice for phenotyping of peripheral blood using our cytotoxic assay, and with Anne McLaren. Anne suggested we look at the other side of the coin, asking whether the positive result we obtained with X/XSxr cells as targets of HY- specific cytotoxic cells could be confirmed by testing for inability to respond to HY. She made a set of experimental mice to test this by mating X/YSxr carrier males with inbred C57Bl/6 females and we skin grafted all the progeny, including X/X females, X/XSxr, XY and X/YSxr males, with B6 X/Y male skin. The result was that only the XX females rejected the test grafts, consistent with HY being a 'self-molecule' expressed by all the males, including the X/XSxr ones, and therefore one to which they could not mount a response (Simpson *et al.*, 1981). This result was consistent with Ohno's hypothesis suggesting the HY was the trigger for male differentiation.

A further experiment done in collaboration with Anne established that the HY epitopes recognised by T cells were different from those by antibodies. We looked at XO females, which had been reported as serologically positive for HY (Engel *et al.*, 1981), (thereafter called SDM, for 'serologically defined male'), and by employing experimental designs similar to those used for identifying the T cell-recognised transplantation antigen, HY, on X/XSxr males, we found that XO females were negative. Their cells were not targets for HY specific cytotoxic T cells and as responders they rejected sygeneic male skin, consistent with their failure to express HY as a self molecule (Simpson *et al.*, 1982).

Interlude with Lamarck

At the same time that we were using genetically determined self-tolerance to test for the presence or absence of the HY transplantation antigen, London was visited by another young scientist interested in self/non-self discrimination. Ted Steele was an Australian who had spent a frenetic post-doctoral year in a Canadian laboratory where he did work leading to a claim that neonatal tolerance to MHC-mismatched antigens induced using Medawar's original protocol could be transmitted to progeny of tolerant male mice. This heterodox return to Lamarckianism raised a few eyebrows, but Peter Medawar himself welcomed Ted to his laboratory to see if he could repeat the contentious results alongside Leslie Brent (then Professor of Immunology at St Mary's Medical School), who had done the original *in vivo* experiments with Medawar (Billingham *et al.*, 1953) and with me, since I had later analysed this model *in vitro* for the development of cytotoxic T cells (Beverley *et al.*, 1973; Simpson *et al.*, 1974). Anne suggested that an extension of this Lamarckian hypothesis would be to test the progeny of tetraparental males, themselves tolerant of both MHC types (Matsunaga *et al.*, 1980), using the same MHC-mismatched strains to be tested for the inheritance of neonatal tolerance. She and I set out to do this in parallel with the experiments being done by Leslie Brent and his colleagues using stud males made tolerant by neonatal injection of MHC-mismatched cells. The results of the combined London experiments, watched over anxiously by Ted Steele, who was also trying to replicate his earlier work, was that we could find no evidence of inheritance of the acquired character of tolerance to transplantation antigens. Anne McLaren shared with Peter Medawar an extraordinarily open-minded approach during this testing time, which ended up with three Nature papers (Brent *et al.*, 1981; McLaren *et al.*, 1981a; Brent *et al.*, 1982) detailing the negative

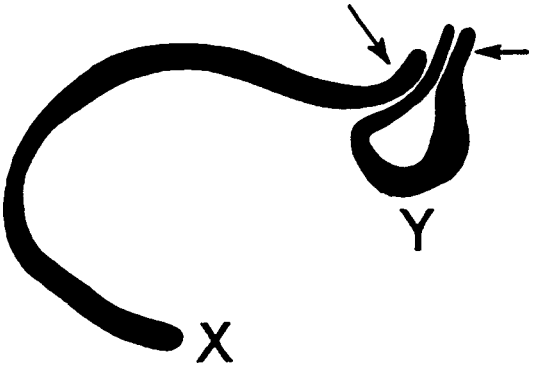

Fig. 1. Meiotic pairing configuration of X and Y Sxr^a chromosomes observed by electron microscopy of testis preparations from an X/Y Sxr^a male. *(modified from Chandley and Speed, 1987, Chromosoma 95: 345-349).*

results (another sort of record) and a great deal of noise from Fleet St, where Ted spent increasing amounts of time drumming up support once the laboratory failed him.

Moving between X and Y

Meanwhile Anne McLaren's imaginative approach had led her into rather different paths, to be joined by me after she and Marilyn Monk had successfully devised a method for investigating the inheritance of Sxr (McLaren & Monk, 1982). For many years Sxr had been assumed to be an autosomal mutation, since it was passed by carrier males to half of their X/X and half their X/Y progeny (X/XSxr developing as sterile males and X/YSxr as carrier males). The possibility that it was X/Y linked by being located on the pseudoautosomal region of the sex chromosomes was explored by crossing X/YSxr carrier males to females carrying Searle's translocation, T(X;16)16H (T16H). Amongst the progeny were fertile T16H/XSxr female mice that could transmit Sxr to male X/XSxr and X/YSxr progeny. This provided evidence that Sxr was X and Y linked. Anne's findings had been replicated in Cattanach's lab (Evans *et al.*, 1982), and those of Singh and Jones (Singh & Jones, 1982) using *in situ* hybridisation were consistent with the same conclusion. The development of T16H/XSxr individuals as females was due to X inactivation of the maternally derived T16H chromosome (consisting of part of chromosome 16 and part of the X) being a cell lethal event, so the embryo consisted only of cells which had inactivated the paternally derived XSxr. Since Sxr carried the testis determining gene, now identified as Sry, (Koopman *et al.*, 1991) it was assumed that this also was inactivated during the crucial stage of gonadogenesis.

Serendipitous discovery of Sxr^b: Ohno's hypothesis revisited

It was a small step then to ask questions about HY expression of T16H/XSxr female mice, but a crucial one to test Ohno's hypothesis further. That such females did express might be construed as a falsification of the hypothesis, except that it could be argued that expression in adult tissues might not have been mirrored in the foetus at the time the gonads were developing, thus allowing ovarian rather than testicular differentiation. Nevertheless, initially Anne and I chose to test for HY expression progeny

inheriting Sxr from either the father (X/YSxr or XSxr/YSxr) or the mother (T16H/XSxr). These Sxr carrier mice had been generated during the work cited above (McLaren & Monk, 1982), but had the disadvantage for me, as an immunologist, of having come from outbred stock, in which several different H2 haplotypes were segregating. This meant we had to H2-type the mice in order to choose the right HY-specific, H2-restricted typing T cells. Our first experiment identified the presence of $H2^b$ or $H2^k$ haplotypes in most of the mice, and subsequently we found that $H2^q$ was present in others (Simpson *et al.*, 1986). In our first experiment all the T16H/XSxr females bar one typed positive for HY with the appropriate cells (Simpson *et al.*, 1984), and many dozens more typed positive in experiments over the next several months. Samples of spleen were taken by partial splenectomy for H2 and HY typing so that mice of potential interest could be retested or bred if relevant. This was obviously necessary for the single female we had found HY-negative, since we needed to discover whether this characteristic was shown by males inheriting Sxr from her, to determine if a further mutation in it had occurred, rather than an epigenetic modulation of expression in this one individual.

We waited many months for the informative progeny. One was an X/XSxr son (necessarily sterile), also HY-negative. More important for establishing the putative mutation in a breeding stock was an XSxr/Y son, who fathered not only HY-negative X/XSxr males but also a negative T16H/XSxr female, through which the mutant Sxr could also be transmitted (McLaren *et al.*, 1984). These results conclusively showed that male sexual differentiation could take place in mice lacking the expression of HY antigen, therefore disproved Ohno's hypothesis. Studies of cell lines from patients with abnormal Y chromosomes confirmed the separation of the Y chromosome gene determining testis differentiation and that controlling HY expression in humans (Simpson *et al.*, 1987).

Our mouse mutation, subsequently named Sxr^b to distinguish it from the Sxr^a from which it was derived, had affected or removed the HY gene(s). It had also, as shown by Paul Burgoyne, removed a gene or genes controlling crucial steps in spermatogenesis (Burgoyne *et al.*, 1986), thus making HY gene(s) candidates for this male-specific role. The extent to which HY expression had been obliterated in Sxr^b was also shown *in vivo*, with the grafting approach Anne and I had used earlier for HY-typing X/XSxr males and XO females (Simpson *et al.*, 1981; Simpson *et al.*, 1982). Not only did X/$XSxr^b$ males reject skin grafts from otherwise histocompatible XY males (Simpson *et al.*, 1986), but spleen cells from X/$XSxr^b$ males failed to immunise XX females to reject syngeneic male skin grafts, whereas those from X/$XSxr^b$ and X/Y males did so (McLaren *et al.*, 1988a).

Having discovered one HY-negative T16H/XSxr female in our first experiment, in which only eight such mice were examined, we thought that more were likely to appear amongst similar stocks in which Sxr^a was segregating. So week after week Anne and I did partial splenectomy on this stock, as well as the backcross stock she was creating to take the Sxr^b mutation onto the C57Bl/6 strain, to fix the H2 haplotype. Since we did all our experiments in a coded manner, I had no record of the phenotype or genotype of the mice from which the samples were taken, until I had completed the H2 and HY typing of each weekly batch of between 20 and 30 mice. Once that was done, we put together the information each of us had, to identify further possible mutations or recombinations. On several occasions we came across apparently unexpected HY-typing results, only to find them not reproducible on subsequent partial splenectomy samples, or that Anne had by palpation mistaken an X/Y for an X/XSxr male: this was sorted out histologically.

One reproducible result however was that of a single HY-positive X/XSxr male in the Sxr^b backcross. This was an important finding, assumed to be the result of an illegitimate X-Y crossover involving the replacement during meiosis of the mutant Sxr^b on the pseudoautosomal portion of the carrier male Y chromosome, with the short arm of the same Y chromosome (see Fig. 1 to illustrate this configuration). It provided direct evidence for the mapping of Sry and the HY gene(s) onto the short arm of the normal Y chromosome (McLaren *et al.*, 1988b). It also suggested that the original Sxr^a mutation might have arisen by a similar illegitimate recombination (see Fig. 2 for a diagrammatic account of this event), and that such events might be not that uncommon, if one set out to look for them. Bruce Cattanach, who had discovered the original Sxr^a, did this by breeding from $XSxr^a$/Y males (Capel *et al.*, 1993). Anne had earlier initiated a set of experiments using a different approach to obtain new recombinant Sxr regions, by mating an $XSxr^b/YSxr^a$ male to X/X and X/O females. A number were obtained, identified by phenotyping the progeny for HY expression and spermatogenesis, and probing for Y chromosome sequences which also distinguished Sxr^a from Sxr^b (McLaren *et al.*, 1992).

Immunogenetics of Sxr

The Sxr^b mutation was found by my namesake, working in David Page's laboratory, to be the result of a gene fusion between two duplicated genes, *Zfy1* and *Zfy2* (Simpson & Page, 1991). The human homologue, ZFY, had been discovered in the Page laboratory during the search for the human testis determining factor, TDF, to which it lay confusingly close (Page *et al.*, 1987). But if ZFY was a cause of some anguish, on account of this unfulfilled role, the mouse homologues, Zfy1 and Zfy2, provided crucial mapping data

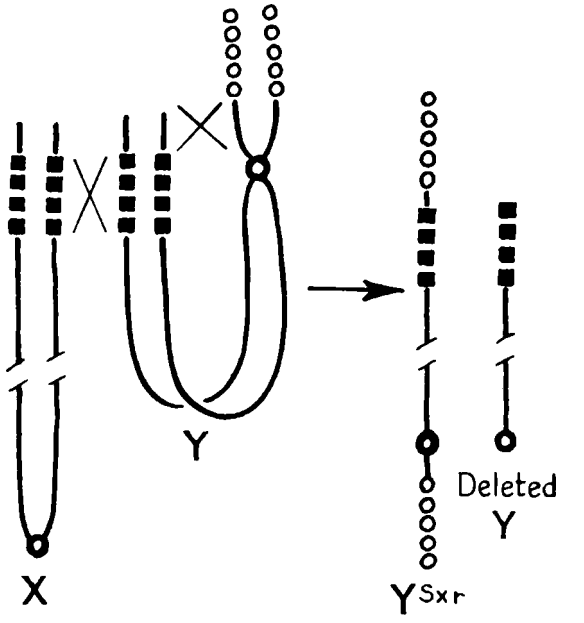

Fig. 2. Proposed origin of the original Y Sxr^a chromosome, from an anomalous interchromatid exchange in a normal Y *(from McLaren et al., 1988b).*

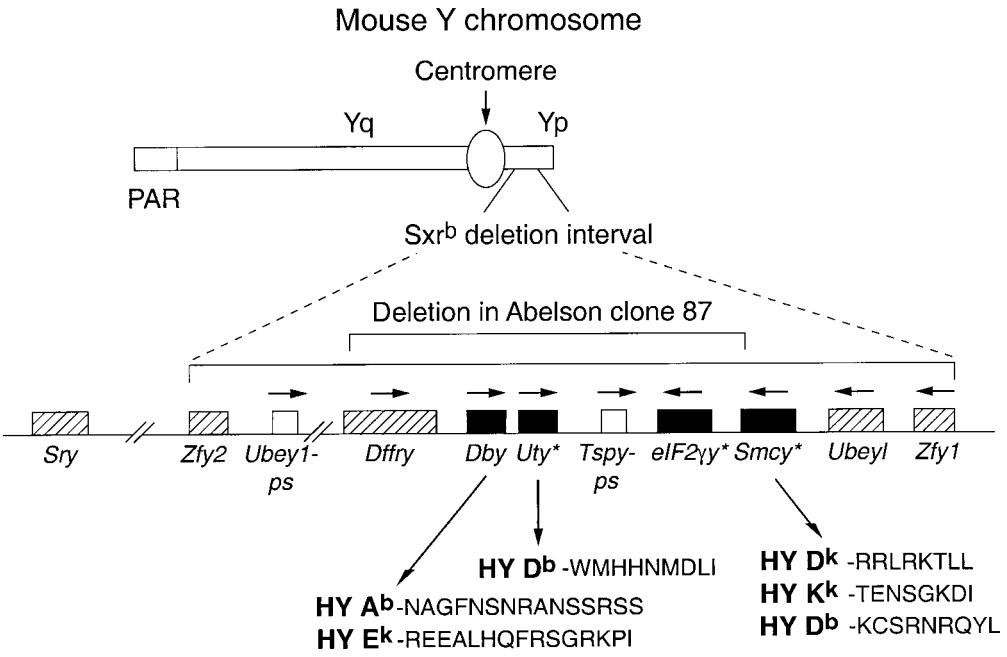

Fig. 3. Schematic representation of the mouse Y chromosome. This sh*ows the location of the Sxr^b deletion interval on the short arm, Yp, mouse Y chromosome and a transcription map of Sxr^b. The genes are represented by boxes: open for non-functional genes, hatched for testis-specific and black for ubiquitously expressed genes. Their orientation is shown by the arrows pointing towards the 3' end. The asterisks indicate genes whose X chromosome homologues are known to escape X-inactivation. Above is shown the extent of the deletion in Abelson clone 87. The HY epitopes expressed by the genes are given below.*

for the gene(s) encoding or controlling expression of HY antigen in the mouse. They had been removed in the gene fusion event which gave rise to Sxr^b.

Several parallel approaches were made to home in on identifying the HY gene(s). We had obtained indirect evidence that there was more than one gene, in that individual clones of an Abelson-transformed cell line from a T16H/X Sxr female expressed variable numbers of HY epitopes, with HY/D^b absent from many clones whilst present in others (Scott *et al.*, 1991). This suggested the variable spread of X-inactivation into the translocated segment, in which it would reach one but not other HY genes. One approach to confirm this directly, and to map the genes in relation to each other within the Zfy1-Zfy2 deletion interval (ΔSxr^b) was to use the immunoselection method pioneered by Derry Roopenian at the Jackson laboratory. The starting point for this work was another Abelson-transformed cell line, this time from an XSxr^a/O mouse heterozygous for H2^k and H2^b, the haplotypes for which we had the relevant HY-specific T cells. These included cytotoxic HY/H2D^k and HY/H2D^b effector T cells, which were used by incubating each separately with an aliquot of cloned XSxr^a/O Abelson cells, followed by the recovery of those Abelson cells that had survived the selection procedure. From them, those that had evaded killing by down modulation of the relevant restriction molecules, H2D^k or H2D^b, were discarded, so that those which had escaped by failing to express HY alone could be isolated and tested. These studies included both tests for other HY epitopes expressed by the non-selected clone, with detection of HY/H2K^k and HY/ H2A^b, in addition to the reciprocal of selected epitopes, HY/D^k and HY/D^b, and tests for DNA markers mapped into the deletion interval (King *et al.*, 1994). The results amply confirmed the likely existence of a number of HY genes, which could be individually affected by immunoselection, and suggested a gene order in relation to the rather small number of markers then available within this deletion interval (see Fig. 3 for an updated version of this).

A second approach to gene and subsequent HY peptide epitope identification was expression cloning using HY-specific T cells. The methods for doing this to identify genes encoding MHC class I epitopes recognised by cytotoxic T cells had been established using similar approaches to identify tumour antigens. These had been pioneered by Thierry Boon and his colleagues (Boon *et al.*, 1994), and by model experiments in our laboratory (Scott *et al.*, 1992). Work on the physical mapping of the Srx^b deletion interval by our collaborator Michael Mitchell in Marseille helped to provide candidate genes, including *Smcy,* which was the first gene identified as encoding an MHC class I-restricted HY epitope (HY/H2K^k in mouse) (Scott *et al.*, 1995) and Dby, the first gene identified as encoding MHC class II -restricted minor H epitopes (HY/H2A^b and HY/H2E^k) using a novel expression cloning approach (Scott *et al.*, 2000). Another candidate emerged as a cDNA of Uty, mapping into the Sxr^b deletion interval (Greenfield *et al.*, 1996), and which encodes the immunodominant HY/H2D^b epitope (see Fig. 3). Our work and that of other laboratories, using similar or complementary biochemical approaches, has discovered further HY epitopes encoded by *Smcy* in mouse and by *SMCY, UTY* and *DFFRY* in humans (Warren *et al.*, 2000; Wang *et al.*, 1995; Meadows *et al.*, 1997; Vogt *et al.*, 2000). These human Y chromosome genes, which are closely homologous to their mouse counterparts, map to the long arm, Yq, in a region syntenic with the mouse Sxr^b deletion interval, but not so neatly demarcated by flanking Zfy genes (Lahn & Page, 1997). ZFY in humans is on Yp and is not duplicated (Lahn & Page, 1997; Page *et al.*, 1987).

The physiological function of HY encoding genes, which are ubiquitously expressed, is not understood at present, aside from characterising them by sequence homologies, e.g. part of Smcy/SMCY is homologous to RBP (Scott *et al.*, 1995). Attempts to examine function by deletion using homologous recombination in murine ES cells have been unsuccessful in several laboratories. What also makes these genes and their X homologues intriguing is that the X homologues escape inactivation, implying the need for expression of two copies of each gene, from both X copies in females and the X and Y copy in males.

Translational potential of molecular genetic information on HY

Our laboratory is primarily an immunological one but we became drawn into a number of other areas by hypotheses on the physiological function of the molecule HY. For us HY has been a model 'weak' transplantation antigen, of which minor histocompatibility (H) antigens, tumour antigens and autoantigens are examples. There is a clinical need to be able to immunomodulate responses to H antigens following the grafting from genetically dissimilar donors. For example solid organ transplantation, e.g. kidney, which elicits a host-versus-graft (HVG) response unless the patient is treated with immunosuppressive drugs, which interfere with protective responses to pathogens and to incipient tumours. In the example of transplanting bone marrow haematopoietic stem cells into genetically non-identical recipients, HVG responses can interfere with engraftment, but the additional complication of graft-versus-host (GVH) responses can also threaten the recipient, and require immunosuppressive treatment. For autoimmune diseases control of harmful immune responses to self molecules, whilst maintaining protective responses to pathogens is also desirable. For tumour patients, in contrast, there is a need to be able to upregulate immune responses to antigenic epitopes selectively expressed on tumours.

A crucial step forward is enabled once the peptide epitopes recognised by effector T lymphocytes are identified, since they provide the means both to monitor and modulate immune responses. For monitoring, fluorescent tetrameric complexes made from the appropriate recombinant MHC restriction molecules folded with β_2-microglobulin and the peptide, allow the staining of HY-specific T cells. This includes both those cultured *in vitro* and those freshly taken *ex vivo* from a mouse or patient making an anti-HY response (Mutis *et al.*, 1999; Millrain *et al.*, 2001). Other specific immunological tests such as those detecting cytokine production at the single cell level can also be carried out using the cognate HY peptides (or those of other, autosomally-encoded minor histocompatibility antigens).

New approaches to inducing graft donor-specific tolerance have also been opened up by the identification of minor H peptides, as exemplified by the male-specific HY epitopes. Earlier work in our laboratory had shown that exposure to tissues expressing a single $CD8^+$ T cell-recognised peptide epitope, in the absence of 'helper' epitopes to which CD4 T cells could respond, resulted in tolerance to subsequently placed skin grafts bearing both cytotoxic and helper epitopes (Antoniou *et al.*, 1996). This has now been extended by the use of class I-restricted HY peptides pulsed onto syngeneic female dendritic cells to induce tolerance in female mice to subsequently placed male skin grafts expressing both those CD8-recognised peptides and additional CD4-recognised epitopes. The induction by the initial class I-restricted peptide of regulatory T cells appears to be a likely mechanism (James *et al.*, 2001). The possibility of extending this 'bystander tolerance' to additional transplantation antigens in the clinical setting is possible, and the results of Waldmann and Cobbold (Cobbold *et al.*, 1996), working in mice with molecularly undefined multiple minor antigens would also imply this.

Anne: the role model, scientist, colleague and friend

The hypothesis about the role of HY in sex determination provided a starting point for the experiments I did with Anne. Whilst they resulted in the disproof of a very interesting theory, they led to our being able to get a handle on identifying the genes encoding HY epitopes, by virtue of the immunological, genetic and molecular characterisation carried out on the Sxr^b mutation. Anne was a crucial player in all of that work. Her interest in immunology provided common ground which took us into enquiries about HY expression in X/XSxr mice, and her understanding of the genetics of mouse development led me on into areas in which I would not have ventured without her. I suspect our laboratory would not have cloned the HY genes or been placed in a position where we can now exploit the information gained, both to explore fundamental aspects of the control of immune responses, and in translational approaches.

Anne is not just a mentor and colleague with whom I have enjoyed a productive working relationship. She is also a friend and a very important role model as a woman scientist, who has a mind as sharp as anyone, and who uses it as an instrument of rational enquiry. She has managed to juggle manifold demands of students, colleagues, friends and family, as well as wider demands in the scientific community. The fact that she is a woman does not make that a more remarkable feat – as she once said to me, women used to coping with small children have the knack of doing several things simultaneously. She said this when I had a small child 'at foot', and I realised the truth of it, and it was both funny and instructive. It has certainly influenced the way I work, and my expectations, of myself and of my younger colleagues and students.

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