A search for sex-specific antigens on bovine spermatozoa using immunological and biochemical techniques to compare the protein profiles of X and Y chromosome-bearing sperm populations separated by fluorescence-activated cell sorting

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Currently, the only successful method for separating X and Y chromosome-bearing spermatozoa is fluorescence-activated cell sorting. Although effective, this technique is of limited usefulness to the animal breeding industry as it cannot produce the large volumes of sexed spermatozoa needed for artificial insemination. An attractive alternative would be to identify an immunological marker confined to one sperm type and, therefore, significant scientific effort has been expended in examining antibodies that appear to recognize approximately 50% of spermatozoa in an ejaculate. However, no sex-specific antigens have yet been identified from spermatozoa. Using the opportunity afforded by the development of sperm separation by fluorescence-activated cell sorting, we have made a thorough search for differences between X and Y chromosome-bearing bull spermatozoa using both biochemical and immunological methods. Techniques for radiolabelling surface membrane proteins, in conjunction with SDS-PAGE, failed to show any differences between populations. Similarly, a wide range of monoclonal antibodies raised to ejaculated, cauda epididymal and testicular spermatozoa failed to distinguish between the X and Y chromosome-bearing spermatozoa. Only after analysis by high resolution two-dimensional SDS-PAGE was an indication obtained that X-specific proteins occur. However, these proteins are not associated with the surface membrane and further work is necessary to confirm their association with the X chromosome and to characterize them more fully. Our inability to detect sex-specific differences in sperm surface antigenicity suggests that further work on this immunological approach to semen sexing is unlikely to be profitable.

Introduction

A reliable method for controlling the sex of farm animals would have considerable impact on the efficiency of the animal breeding industry and, if applicable to humans, could also be used to reduce the incidence of sex-linked diseases. An obvious way of achieving this is to separate X and Y chromosome-bearing spermatozoa before insemination. In the past, a number of methods for sperm separation have been devised based on supposed differences in density (Harvey, 1946), speed of swimming (Ericsson *et al.*, 1973), surface charge characteristics (Kaneko *et al.*, 1984), interaction with H-Y antibody (Goldberg *et al.*, 1971) and staining response with quinacrine (Barlow and Vosa, 1970; Pearson and Bobrow, 1970). Thus, the procedure of allowing spermatozoa to distribute through a column of albumin, claimed to bias the outcome of pregnancy in humans, is based on a belief that Y chromosome-bearing spermatozoa

are more motile than X chromosome-bearing spermatozoa (Beernink *et al.*, 1993). However, the albumin technique, in common with other sperm separation methods based on apparent inequalities between X and Y chromosome-bearing spermatozoa, has produced equivocal results (reviewed in Windsor *et al.*, 1993).

The only feature of mammalian spermatozoa currently accepted as differing consistently between the X and Y chromosome-bearing components of the population is DNA content. In mammals, the X chromosome is larger than the Y chromosome and hence contains more DNA. The degree of difference depends upon species and amounts to approximately 2.9% in human spermatozoa (Sumner and Robinson, 1976), approximately 3.8% in bulls and as much as about 7.5% in chinchilla (Johnson, 1992). This disparity in DNA content has been exploited to develop a successful method of sperm separation using flow cytometry. After exposure of spermatozoa to Hoechst 33342, a fluorescent dye that binds stoichiometrically to DNA, two populations can be distinguished on

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the basis of their fluorescent characteristics, and these can be separated by fluorescence-activated cell sorting (FACS) using a modified flow cytometer (Johnson and Pinkel, 1986). In this way, spermatozoa from rabbits (Johnson *et al.*, 1989), pigs (Johnson, 1992), bulls (Cran *et al.*, 1993) and humans (Johnson *et al.*, 1993) have been separated into X and Y chromosome-bearing populations with an efficiency of between 70% and 90%. The technique has been validated both by re-analysis of sorted spermatozoa (Johnson *et al.*, 1989) and by sexing individual sorted spermatozoa by PCR (Welch *et al.*, 1995) or *in situ* hybridization with X or Y chromosome-specific DNA probes (Johnson *et al.*, 1993). In addition, fertilization trials with sorted spermatozoa in rabbits (Johnson *et al.*, 1989) and cattle (Cran *et al.*, 1993) have produced appropriately biased sex ratios and have also shown that separating spermatozoa by FACS does not adversely affect their fertilizing capacity or prevent normal embryonic development. A study in rabbits, however, suggested an increase in the number of fetuses lost during the early stages of pregnancy (McNutt and Johnson, 1996). The technique is regarded as suitable for use in humans and has been used in at least one clinical trial to prevent the possibility of X-linked hydrocephalus (Levinson *et al.*, 1995).

Although clearly effective, the use of FACS as a routine tool for sex selection in animal breeding is limited by the low rate of sorting (about $0.5 \times 10^6 \text{ h}^{-1}$), which produces too few spermatozoa for standard artificial insemination (AI) procedures and restricts its use to *in vitro* fertilization techniques. However, the use of FACS provides a new opportunity to investigate and compare separated populations of X and Y chromosome-bearing spermatozoa for properties that might be of use in designing methods to separate large numbers of spermatozoa for AI. In this respect, the surface components of the two populations are of particular interest, since differences in membrane antigenicity could be exploited to generate antibodies capable of separating X or Y chromosome-bearing spermatozoa.

We have investigated the profile of antigens in both unsorted and FACS-sorted bovine spermatozoa using a variety of techniques including radiolabelling of surface membrane components, one- and two-dimensional gel electrophoresis of sperm proteins and reactivity with a large panel of polyclonal and monoclonal antibodies to sperm proteins. The aim has been to look for a consistent difference between X and Y chromosome-bearing spermatozoa that could form a reliable basis for developing immunological separation techniques.

Materials and Methods

Chemicals

All chemicals were of the highest purity commercially available and were obtained from Sigma Ltd (London), Pharmacia Biotech (St Albans, Herts) or BioRad Laboratories Ltd (Hemel Hempstead, Herts). Reagents for two-dimensional SDS-PAGE and 'Immobilon' blotting membrane were purchased from Millipore (UK) Ltd (Watford, Herts). Bolton and Hunter reagent and Na^{125}I were supplied by Amersham International (Little Chalfont, Bucks), Iodogen reagent by Pierce and Warriner (UK) Ltd (Chester, Cheshire).

Collection and washing of semen

Semen was collected using an artificial vagina from Holstein Friesian bulls of proven fertility. Care was taken to avoid exposing the semen to temperature changes during its transfer to the laboratory, which occurred within 1.5 h of collection. Semen was washed by diluting 1:1 with PBS and layering samples of about 3 ml over a cushion of $0.25 \text{ mol sucrose l}^{-1}$ in $10 \text{ mmol Tris-HCl l}^{-1}$, pH 7.4, in a glass centrifuge tube. The sample was centrifuged at $450 g$ for 10 min at room temperature and the supernatant aspirated to leave a loose pellet of spermatozoa. Seminal plasma adhering to the walls of the tube was wiped away with a tissue; the sperm pellet was resuspended in 9 ml PBS, centrifuged as above and the pellet made up to a final volume of 1 ml in PBS.

Testicular and epididymal spermatozoa were collected post mortem from bulls. The testes were transported to the laboratory within 2 h of removal and spermatozoa were flushed out of the cauda epididymidis with PBS. Testicular spermatozoa were collected by aspiration of testicular fluid from the rete testis. Cauda epididymal spermatozoa, which were uncontaminated with other cell types, were diluted tenfold in PBS and washed twice by centrifugation as described above. Rete testis fluid, which contained variable amounts of red blood cells and ciliated epithelial cells, was layered over a three-step Percoll gradient (20%, 40%, 60%) and centrifuged at $2000 g$ for 20 min. Spermatozoa accumulating at the 40%/60% interface were removed and washed once in PBS as described above.

Separation of X and Y chromosome-bearing spermatozoa by fluorescence-activated cell sorting

A washed sample of spermatozoa ($2 \times 10^7 \text{ ml}^{-1}$) was incubated with $10 \mu\text{mol Hoechst } 33342 \text{ l}^{-1}$ for 1 h at 35°C . The stained spermatozoa were then passed through a fluorescence activated cell sorter (FACStar Plus; Becton Dickinson) fitted with an ultraviolet laser and with two photomultiplier tubes placed at right angles to detect sperm orientation, as described in Cran *et al.* (1993). The fluorescence signal from appropriately aligned spermatozoa was used to identify them as X or Y chromosome-bearing as they passed the 0° detector and the cells were subsequently deflected into two separate sterile 2 ml polypropylene tubes (Falcon 2063). The sorting rate was approximately $0.5 \times 10^6 \text{ cells h}^{-1}$ of each type. The introduction of extraneous proteins to the sperm surface was avoided by not using bovine serum albumin in the sheath fluid and not collecting the spermatozoa into egg yolk (standard procedure when collecting spermatozoa for fertilization) but to optimize recovery of sorted spermatozoa, tubes were pre-coated with 0.1% FCS in PBS, thoroughly rinsed with PBS and dried. At intervals during the sorting process, spermatozoa in the collecting tubes were concentrated by centrifugation since they were observed to become immobile at high dilutions.

Examination of sperm surface proteins by one-dimensional SDS-PAGE

The small number of spermatozoa derived from cell sorting precluded direct comparison of detergent-extracted proteins

from X and Y chromosome-separated populations by SDS-PAGE, even using relatively sensitive silver staining techniques. Hence, surface membrane proteins were radiolabelled with [125 I] to increase sensitivity of detection. For labelling surface proteins, samples of sorted ejaculated spermatozoa containing 2.5×10^6 cells were iodinated using either Iodogen reagent (Markwell and Fox, 1978) or the lactoperoxidase technique (David and Reisfeld, 1974) using protocols described by Jones *et al.* (1983). Samples of unsorted spermatozoa (3.5×10^8 cells) from ejaculates and cauda epididymides were similarly treated. For comparative purposes, unsorted spermatozoa were also iodinated using Bolton and Hunter reagent (Bolton and Hunter, 1973) which iodates a wider range of proteins than the other methods, but does not selectively label those at the cell surface. Proteins were extracted from the washed, radiolabelled sperm pellet by incubation for 4 min in 20 μ l 2% (w/v) SDS in 62.5 mmol Tris-HCl l^{-1} , pH 7.4. Solubilized proteins were collected by centrifugation at 10 000 g for 5 min and stored at $-20^\circ C$. Accrued samples from four separate sorts, using material from three different bulls, were combined to give sufficient material to reach detection amounts. Proteins were precipitated with 9 volumes of ice-cold 100% ethanol for 30 min, the pellets dried and solubilized by boiling in 100 μ l 2% SDS in 62.5 mmol Tris-HCl l^{-1} , pH 7.4 before being loaded on to 8.5–12.5% gradient gels to give 100 000 c.p.m. in each well. After electrophoresis, gels were fixed (40% methanol, 7% acetic acid, 53% water), dried and radioactivity detected with Hyperfilm-MP (Amersham) at $-80^\circ C$.

Production of monoclonal antibodies

Whole spermatozoa or membrane vesicles resuspended in PBS were emulsified with complete Freund's adjuvant and injected subcutaneously at multiple sites into female Lou/C rats (Harlan Olac, Bicester). A second injection of the antigen in incomplete Freund's adjuvant was given 30–40 days later. Preimmune and immune blood was collected from each rat.

Groups of three rats were immunized using a number of different protocols. These were: (1) washed, unsorted ejaculated spermatozoa in numbers ranging from 2.5×10^6 and 1×10^7 cells per rat; (2) X or Y chromosome-enriched populations of ejaculated spermatozoa at 4×10^6 cells per rat; (3) an enriched sperm plasma membrane preparation obtained from ejaculated spermatozoa by vortexing (Jones, 1986); (4) as in groups 1–3 above using cauda epididymidal spermatozoa; (5) unsorted or FACS-sorted testicular spermatozoa; and (6) initial immunizations with unsorted cauda epididymidal or testicular spermatozoa, or with enriched plasma membrane preparations of cauda epididymidal spermatozoa, followed by a booster injection of sorted ejaculated spermatozoa.

Group 1 immunizations were designed to assess the numbers of spermatozoa needed to produce a strong immune reaction. Plasma membrane preparations were used in an attempt to target the production of antibodies to membrane-associated proteins; that is, those most likely to be of use for semen sexing, while spermatozoa from the testis and cauda epididymidis were used to avoid the highly antigenic seminal plasma proteins abundant on the surface of all ejaculated spermatozoa.

Animals showing a strong immune response (assessed using antisera to probe western blots of sperm proteins) were boosted by an intravenous injection of antigen, and 4 days later spleen cells were taken for fusion with either Y3Ag1.2.3 (Galfre *et al.*, 1979) or IR983F (Bazin, 1982) rat myeloma cells, as described by Galfre and Milstein (1981). Supernatants from the resulting hybridomas were screened by western blotting and by indirect immunofluorescence (IIF) on smears of testicular, epididymidal and ejaculated spermatozoa. Antibodies of interest were cloned from single cells using the limiting dilution technique.

Screening of antisera and hybridoma supernatants

Antisera and supernatants were screened on western blots (Jones, 1986) containing detergent-extracted proteins (1% (v/v) Nonidet P-40 (NP40) in 50 mmol Tris-HCl l^{-1} , pH 7.4, for 30 min at $4^\circ C$) from ejaculated or cauda epididymidal spermatozoa separated by SDS-PAGE (Laemmli, 1970). Where appropriate, gels were stained with 5% (w/v) Coomassie blue R-250 in methanol: acetic acid: water (40:7:53). Protein concentrations were measured by absorbance at 280 nm (E_{280} of 1.0 taken as equivalent to 1 mg ml^{-1}) or colorimetrically by dye-binding (Bradford, 1976) using BSA as standard. In addition, all supernatants, irrespective of their reactivity on western blots, were screened by indirect immunofluorescence (Gaunt *et al.*, 1983) on air-dried smears of unsorted or sorted spermatozoa. Where appropriate, additional checks were made using washed, intact spermatozoa labelled in suspension. The cellular location of antibody binding and the percentage of fluorescent spermatozoa were calculated from counts of 300 cells.

High resolution two-dimensional SDS-PAGE

Sperm samples of very high purity separated by FACS were obtained by reducing the width of the sort gates (see Fig. 1b) to collect only from the extreme edges of the population. Although this restricted the rate at which spermatozoa could be accumulated, it increased the purity of the samples to about 80%. In three separate experiments, sperm samples from 12–19 individual sorts of 1 day duration were pooled to give sufficient material for two-dimensional SDS-PAGE analysis. In one of these experiments semen from three different bulls was used, while in the other two experiments the pooled samples were from a single bull but from a different individual in each case. Each sorted sperm sample was transferred to an Eppendorf tube and centrifuged at 9000 g for 10 min, the supernatant was removed and the sperm pellet frozen at $-20^\circ C$. When sufficient material had been accumulated for each experiment, the samples were pooled into a total volume of 20 μ l of lysis buffer (9 mol urea l^{-1} , 2% (w/v) NP40, 20 mmol DTT l^{-1}) for 1 h at room temperature. The lysate was then centrifuged at 9000 g for 10 min and the supernatant retained on ice until loaded onto the isoelectric focusing (IEF) gel.

Two-dimensional SDS-PAGE was carried out using the Millipore InvestigatorTM system following the manufacturer's instructions. Briefly, for IEF a mixture of broad range (pH 3–10) and narrow range (pH 4–8) ampholytes was included in a gel solution containing 9.5 mol urea l^{-1} , 2% NP40,

Fig. 1. Identification and separation of X and Y chromosome-bearing spermatozoa from a bovine ejaculate. (a) Two-dimensional plot of fluorescence monitored by 90° and the 0° detectors, showing the appearance of two peaks within the high fluorescence region of the 90° detector. The lower peak is characteristic of Y chromosome-bearing spermatozoa aligned edge on to the 90° detector, while the upper peak derives from X chromosome-bearing spermatozoa. (b) Histogram of these two peaks showing the degree of overlap between the two populations and the positions of the sort gates (M1 for X-bearing and M2 for Y-bearing spermatozoa) controlling the regions of selection within the peaks.

4.1% acrylamide and 5 mmol CHAPS l^{-1} cast in pre-threaded tubes (internal diameter, 1 mm). The tubes in the first-dimension running tank were positioned vertically with the lower end in anode buffer (100 mmol phosphoric acid l^{-1}) and the upper in cathode buffer (100 mmol NaOH l^{-1}). After prefocusing for 90 min, samples were injected on to the gel surface on the cathode side. Extracts from X and Y chromosome-bearing samples were run in parallel and all samples were run under identical conditions (18 000 Volt-hours: 17 h at 1000 V, 0.5 h at 2000 V) so that gels would be comparable. The focused tube gels were extruded into 5 ml gel equilibration buffer (0.3 mol Tris base l^{-1} , 0.075 mol Tris-HCl l^{-1} , 3% (w/v) SDS, 50 mmol DTT l^{-1} and 0.01% (w/v)

bromophenol blue), incubated at room temperature for 2 min and transferred to the top of the second dimension gels. These were slab gels (230 mm × 220 mm × 1 mm) designed to give maximum resolution of proteins and were cast using Duracryl (Millipore), a high tensile strength acrylamide, at a final concentration of 12.5%. Gels were subjected to electrophoresis together in a temperature-controlled tank at 20°C for 5–8 h at 16 000 mW per gel. Gels were silver stained according to the protocol recommended by Millipore based on the technique of Morrissey (1981). The positions of all resolved proteins were marked on transparent overlays and these were used to compare the distribution of proteins between gels. Gels were calibrated using two-dimensional SDS-PAGE protein standards (Bio-Rad; pI range 4.5–8.5, molecular mass 76–17.5 kDa).

Results

Efficiency of separation of X and Y chromosome-bearing spermatozoa by fluorescence-activated cell sorting

The efficiency of separation of X and Y chromosome-bearing spermatozoa by FACS is shown (Fig. 1). The high fluorescence signal produced when the flattened heads of bull spermatozoa are viewed edge on by the 90° detector enabled appropriately orientated spermatozoa to be selected for identification as X or Y chromosome-bearing by the 0° detector. The contours in Fig. 1a show that two peaks can be resolved in this region of high fluorescence with the upper peak resulting from the enhanced fluorescence characteristic of spermatozoa carrying the X chromosome. A histogram of the signals from the 90° detector (Fig. 1b) shows these overlapping populations more clearly, together with the positioning of the electronic 'gates', which restricted collection of spermatozoa to the most widely separated edges of the populations. Re-analysis of sorted sperm populations was used to check the efficiency of sorting, and enrichment of between 70% and 80% was achieved

Assessment of variation among bulls in sperm proteins analysed by SDS-PAGE

Detergent-extracted proteins prepared from ejaculated spermatozoa from four different bulls, separated by non-reducing SDS-PAGE and stained with Coomassie blue, showed no differences in protein profile among bulls (data not shown). Similarly, protein profiles of spermatozoa from the rete testis or the cauda epididymides of a number of bulls were comparable within each region. On the basis of these results, it was thought unnecessary to use the same bull for all screening procedures so sperm protein extracts from nine individuals were used to prepare western blots for antibody screening. Two bulls consistently produced semen containing a high percentage (> 75%) of motile spermatozoa, and semen from these animals was used for FACS-sorting.

Radiolabelling of sperm proteins

The profiles of ^{125}I -labelled sperm proteins varied depending upon the method of iodination used (Figs 2 and 3). Bolton and

Fig. 2. Autoradiograph of sperm proteins radiolabelled with [125 I] using (a) permeant Bolton and Hunter reagent and (b) surface labelling Iodogen reagent separated by SDS-PAGE. Both ejaculated and cauda epididymal sperm proteins show similar profiles with Bolton and Hunter reagent, but Iodogen gives heavy labelling of a seminal plasma protein (about 20 kDa) on ejaculated spermatozoa. The pattern of labelling of proteins from epididymal spermatozoa differs between the two reagents with increased labelling of low molecular mass proteins with Bolton and Hunter reagent. Relative molecular mass is indicated in kDa.

Hunter reagent, which is a permeant compound, labelled proteins with a wide range of M_r values and showed strong labelling of a number of components of low molecular mass (< 20 kDa) from both ejaculated and epididymal spermatozoa (Fig. 2). The Iodogen and lactoperoxidase techniques, which restrict radiolabelling to proteins at the cell surface, resulted in very heavy labelling of a group of seminal plasma proteins at 18–20 kDa which tended to dominate autoradiographs of gels of ejaculated spermatozoa and obscure more weakly labelled protein bands (Fig. 2, lane 6). The association of radioactivity with a whole range of proteins after Iodogen treatment can, therefore, be seen more easily in extracts from

Fig. 3. Autoradiograph comparing surface protein profiles of FACS-sorted and unsorted ejaculated spermatozoa radiolabelled using (a) Iodogen reagent or (b) lactoperoxidase. Extracts of both X and Y chromosome-bearing spermatozoa have identical profiles and differ from unsorted spermatozoa only in the reduced presence of a heavily labelled protein of about 20 kDa derived from seminal plasma. This band is so predominant in the lactoperoxidase labelled unsorted sperm extract that other labelled proteins are underexposed. Relative molecular mass indicated in kDa.

epididymal spermatozoa, which are free of seminal plasma (Fig. 2). Since the epididymal proteins shown (Fig. 2) come from two different bulls, this serves to illustrate the similarity in protein profiles between individuals previously noted for preparations stained with Coomassie blue.

Both methods of surface labelling resulted in identical profiles for the X and Y chromosome-enriched populations of ejaculated spermatozoa after FACS separation (Fig. 3). However, a comparison of the three tracks in Fig. 3a shows that the sorted populations differ from unsorted spermatozoa both in weaker labelling of the 18–20 kDa seminal plasma bands and heavy labelling of low molecular mass components, reminiscent of the labelling seen with Bolton and Hunter reagent (Fig. 2). This observation suggests that the sorting process modifies the sperm surface membrane in two respects; first, it removes loosely bound components; and second, it may cause sufficient membrane damage to permeabilize the cells and expose internal proteins to the Iodogen labelling process. Iodination with lactoperoxidase also failed to reveal any differences between sorted sperm populations (Fig. 3b) and, in the example

illustrated, the very large amounts of labelling associated with the seminal plasma proteins on unsorted spermatozoa again resulted in under-exposure of most other labelled components.

Presence of sex-specific antigens on spermatozoa assessed by immunological techniques

Antisera screening. Individual responses to the different immunization protocols used were assessed by removing a blood sample from each rat after the boosting injection and using the serum to probe western blots of bull sperm proteins separated by non-reducing SDS-PAGE. Most animals showed a strong immune response and all protocols produced antisera that bound to a spectrum of bull sperm proteins ranging from approximately 15 kDa to 200 kDa.

Western blots screened with antisera from group 1 rats immunized with different numbers of spermatozoa showed that, while all rats produced an immune reaction, the strongest response was in animals receiving $> 4 \times 10^6$ spermatozoa. Therefore, in subsequent experiments this was the minimum number of spermatozoa used for immunization. Preliminary western blotting results using antisera from six rats (two immunized with unsorted ejaculated spermatozoa, two with X chromosome-sorted spermatozoa and two with Y chromosome-sorted spermatozoa) showed similar reactions in all rats with only minor variations in the intensity of reaction over certain constituents. One exception to this was a protein of about 50 kDa detected on all blots except those tested with antisera from rats immunized with X chromosome-sorted spermatozoa (data not shown). Two groups of eight rats were identically immunized with either X or Y chromosome-enriched spermatozoa as antigen to establish whether this reflected a real difference in immunogenicity of X chromosome-bearing spermatozoa or resulted from variation in immune response between animals. The first group was injected with sorted spermatozoa derived from ejaculates and the second with sorted spermatozoa from the cauda epididymidis. Results from all 16 animals were comparable. A western blot probed with antisera from the first group showed a considerable degree of similarity between the proteins detected by all eight antisera, again with minor variations, and the protein of about 50 kDa appeared with differing degrees of clarity in all lanes except lane 6 (Fig. 4). Other small variations that occurred between individuals were never associated consistently with either X or Y chromosome-bearing spermatozoa and no antigenic differences between the two types were detected at this level of analysis.

Binding of antibodies to X and Y chromosome-bearing spermatozoa and detergent-extracted sperm proteins separated by SDS-PAGE. Hybridoma supernatants containing anti-sperm immunoglobulins were screened initially using IIF to assess their ability to bind to bull spermatozoa. When antibodies were identified, individual hybridoma cells were cloned and a panel of monoclonal antibodies (mAbs) to a wide range of sperm antigens found on ejaculated, cauda epididymidal and testicular spermatozoa was formed. Many of the mAbs recognized antigens whose distribution was limited to distinct regions of the spermatozoa (the acrosome, postacrosome and equatorial

Fig. 4. A western blot of detergent-extracted ejaculated sperm proteins separated by SDS-PAGE and probed with antisera from eight rats injected with FACS-enriched populations of X or Y chromosome-bearing spermatozoa. No consistent differences between populations were detected. A protein of about 50 kDa (arrowhead), thought from initial experiments to be Y chromosome-specific, was detected by sera from both groups of animals. Lanes 1–4, antisera from rats injected with Y chromosome-bearing spermatozoa; lanes 5–8, antisera from rats immunized with X chromosome-bearing spermatozoa; p, pre-immune serum. Relative molecular mass indicated in kDa.

segment of the head or the midpiece and principal piece of the tail) while others had a more widespread distribution. In all, 15 distinctive labelling patterns could be recognized using antibodies to ejaculated and epididymidal spermatozoa, while only eight of these were identified during the screening of antibodies to testicular spermatozoa. This finding suggests that the maturational changes occurring as spermatozoa pass through the epididymis are accompanied by an increase in sperm antigenicity.

The sperm antigens recognized by different antibodies were identified by screening supernatants from positive hybridomas on western blots of detergent-solubilized sperm proteins separated by SDS-PAGE. A high proportion of the antibodies raised against ejaculated spermatozoa associated with low molecular mass proteins (< 25 kDa). Thus, from a typical fusion, 42% of the supernatant antibodies reacting with

western blots bound to a variety of proteins between 25 kDa and 100 kDa, while 58% bound to just two protein bands at about 18 kDa and 20 kDa. These latter proteins derive from seminal plasma and are present on all ejaculated spermatozoa; they are closely associated with the sperm surface and are not readily removed by washing. The presence of such highly antigenic proteins may mask other, less abundant surface proteins and reduce the likelihood of an immune response being mounted against them. Identical protein blots, probed with antibodies from rats immunized with cauda epididymal or testicular spermatozoa, which have no seminal plasma component, showed a reduction in the number of antibodies recognizing low molecular mass components. In a typical fusion, 70% of antibodies reacted with a variety of higher molecular mass bands and only 30% reacted with proteins at or below 20 kDa.

The extensive panel of mAbs to bull spermatozoa selected during the initial screening process was reviewed to identify potential sex-specific mAbs. On the assumption that equal numbers of X and Y chromosome-bearing spermatozoa are present in semen, 163 mAbs recognizing about 50% of spermatozoa in unsorted smear preparations were identified. When these mAbs were re-tested on smears of sorted spermatozoa, 32 of them bound to a similar proportion (between 40% and 65%) of spermatozoa in both the X and Y chromosome-enriched samples, while the remaining 131 bound to almost all (> 90%) spermatozoa in both samples. One explanation of these results is that the first group of mAbs, which binds to approximately half the spermatozoa in both sorted and unsorted preparations, associates with surface antigens that are not present on all spermatozoa but whose distribution is unrelated to the presence of either sex chromosome. In contrast, mAbs in the second group, where binding to both X and Y chromosome-bearing spermatozoa is greatly increased after sorting, probably recognize internal antigens that are rendered more accessible by the combined processes of flow cytometry and smear preparation. Evidence to support this interpretation was obtained using an antibody, MAC 396, known to bind to an internal antigen. Spermatozoa were labelled in suspension (i) after washing, (ii) after permeabilization by cold-shock (achieved by rapid cooling of washed spermatozoa to 4°C followed by a return to 30°C and a repeat of the cooling process) and (iii) after FACS sorting. Only $23 \pm 4.5\%$ (mean $\pm$ SEM, $n = 3$) of washed spermatozoa bound MAC 396, but this increased to $97 \pm 2.5\%$ ($n = 3$) after cold shock. An intermediate figure of $59 \pm 2.1\%$ ($n = 6$) was obtained for FACS-sorted spermatozoa, which was indicative of a considerable degree of permeabilization during sorting.

High resolution two-dimensional SDS-PAGE

Since differences in sperm surface proteins between X and Y chromosome-enriched populations could not be demonstrated either by immunological means or by using one-dimensional SDS-PAGE, a more detailed comparison of the proteins present in each population was carried out using two-dimensional SDS-PAGE. In this case, lysis buffer was used to make extracts of FACS-sorted spermatozoa so that both surface and internal proteins were included in the analysis. In analysing the paired

gels of X or Y chromosome-derived proteins in each experiment, emphasis was placed on detecting any protein that was consistently present on only one gel in each pair. Smaller quantitative differences that might occur between the gels would not be detected by this method of analysis. Between 234 and 351 protein 'spots' could be resolved by eye, depending on the amount of protein loaded. A spot-by-spot comparison of the protein separations on the X and the Y chromosome-enriched gels showed a close correspondence between the two (Fig. 5a, b). However, in one region a difference was detected, with the presence of a small cluster of proteins (pI about 5.3; molecular mass about 40 kDa) that was unique to the X chromosome-enriched gel (Fig. 5c, d). This group was present on all three of the X chromosome-enriched gels but was not detected on any of the Y chromosome-enriched samples, even when the Y chromosome-enriched sample was more heavily loaded with protein (Fig. 5), thus biasing the system against the appearance of X chromosome-specific proteins. Protein extracts from unsorted spermatozoa and from an unsorted sample enriched with plasma membrane vesicles were run in parallel with the sorted extracts in two experiments. The whole sperm extract gave a virtually identical pattern of protein distribution to that seen in the sorted sperm extracts and a very faint area of silver staining was observed in the 40 kDa, 5.3 pI area of the gel corresponding to the group of proteins of about 40 kDa seen on the X chromosome-enriched gel. Thus, a protein component present in samples containing about 80% of X chromosome-bearing spermatozoa was just detectable in unsorted samples where the proportion of X chromosome-bearing spermatozoa would be expected to be 50%, but was not seen when the proportion of X chromosome-bearing spermatozoa in a sample was reduced to about 20%. The two-dimensional distribution pattern of proteins in the membrane-enriched sample differed from that of the whole sperm extracts, and no component corresponding to the X chromosome-specific group could be detected, which suggested that it was not a surface membrane protein.

Discussion

The presence of an antigen capable of distinguishing between X and Y chromosome-bearing spermatozoa depends upon two suppositions: (i) that expression of genes associated with, or controlled by, the sex chromosomes occurs in haploid spermatids; and (ii) that the gene product is confined to the spermatozoa carrying that chromosome. Although the sex chromosomes have generally been regarded as inactivated by the pachytene stage of meiotic prophase (Handel, 1987), there is increasing evidence for post-meiotic transcription of genes on both chromosomes. Genes on the Y chromosome expressed in post-meiotic spermatids include *Zfy-1* and *Zfy-2* (Nagamine *et al.*, 1990) and *Sry* (Capel *et al.*, 1993), as well as Y353/B, a DNA sequence on the long arm of the mouse Y chromosome (Conway *et al.*, 1994). Two X chromosome-associated genes, *Ube1x* and *MHR6A*, also show pronounced post-meiotic expression (Hendriksen *et al.*, 1995) and there is evidence that the *Hprt* gene remains active during spermatogenesis (Shannon and Handel, 1993). However, while these results confirm that transcripts associated exclusively with either X or Y

Fig. 5. Proteins extracted from FACS-separated sperm populations separated by two-dimensional gel electrophoresis and visualized by silver staining. The gel of proteins from predominantly X chromosome-bearing spermatozoa (a) is less heavily loaded than the gel of Y chromosome-bearing spermatozoa (b) but, nevertheless, a comparison of protein 'spots' appearing on the X chromosome-enriched gel shows that they all have a counterpart on the Y chromosome-enriched gel, with the exception of a group of proteins (pI about 5.3, molecular mass about 40 kDa) within the regions marked on (a) and (b) and enlarged in (c) and (d), respectively. The area of X chromosome-enriched gel shown in (c) contains a group of proteins (arrow) absent from the equivalent area of the Y chromosome-enriched gel arrowed in (d).

chromosome-bearing spermatozoa occur, they may transfer between neighbouring cells by way of the cytoplasmic bridges that link post-meiotic spermatids and become universally distributed (Braun *et al.*, 1989; Caldwell and Handel, 1991).

The present study was designed to look for differences between populations of bovine spermatozoa sorted by FACS by (i) comparing both their surface and intracellular protein complements, and (ii) generating mAbs to a wide range of sperm antigens and assessing their binding to sorted spermatozoa. The first approach is hampered by the very small number of sorted spermatozoa available for analysis. For comparing the surface proteins present in sorted populations, radiolabelling techniques were necessary to allow highly sensitive monitoring of iodinated surface proteins. While separation of iodinated proteins extracted with non-ionic detergents by one-dimensional SDS-PAGE gave identical protein profiles for both sorted populations, it should be borne in mind that only a proportion of surface proteins (specifically those containing exposed tyrosine residues, although histidines and tryptophans are also available for iodination) will be labelled by these methods and the relative amounts of [125 I] incorporated

may not reflect the absolute quantity of protein present. The possibility remains, therefore, of differences between the populations of X and Y chromosome-bearing spermatozoa either in proteins unlabelled by the iodination process or in proteins of low abundance.

Having failed to identify any differences in surface proteins, we separated whole sperm lysates by two-dimensional SDS-PAGE to obtain as complete a picture as possible of the protein complement of the two populations. In this case, a protein cluster specific to the gel of X chromosome-enriched spermatozoa was identified. Three such analyses were carried out with each sample consisting of spermatozoa from between 12 and 19 ejaculates taken from three different bulls. Our results indicate that the X chromosome-specific protein is present consistently in different samples of semen. Its absence from a plasma membrane-enriched preparation suggests that it is intracellular and is, therefore, unlikely to provide a useful marker for sex selection of live spermatozoa. There may be additional differences in protein content between the two populations but these are unlikely to be revealed unless sufficient sorted material is collected to provide a complete

profile of the sperm proteins present in each population that can be subjected to quantitative analysis. This approach has been used to examine X and Y chromosome-enriched samples of boar spermatozoa but no consistent differences between the two populations could be detected (Hendriksen *et al.*, 1996). Even if very large numbers of sorted spermatozoa are accrued, a number of lines of evidence suggest that there is loss of surface membrane components during the sorting process. Thus, radiolabelling studies show both a reduction in seminal plasma proteins associated with the sperm membrane and exposure of internal proteins through changes in membrane permeability to iodinating agents, while antibody studies have shown that the surface membrane of sorted spermatozoa is more permeant than that of unsorted spermatozoa. Membrane damage during sorting might be reduced by using BSA in the sheath fluid, by collecting into a protein-rich medium and by the addition of seminal plasma (Maxwell *et al.*, 1997) but even with these measures in place, our experience suggests that sorted spermatozoa are more fragile than unsorted ones. The modification of the sperm surface suggested by these observations may mean that no analysis of sorted spermatozoa will reflect all the proteins originally present at the surface membrane accurately.

Perhaps the most sensitive probe for detecting differences between cells is the immune response and claims have been made of antibodies able to distinguish between X and Y chromosome-bearing spermatozoa. We have tested populations of X and Y chromosome-bearing bovine spermatozoa separated by FACS with a panel of mAbs that initially were found to bind to about 50% of spermatozoa in unsorted preparations. Subsequently, however, none of the antibodies showed specificity to either X or Y chromosome-bearing spermatozoa. In most cases the initial observation could be explained on the basis of differential access of the antibody to an intracellular antigen, dependent upon the integrity of the sperm surface membrane, but a few examples of antibodies to surface membrane-associated proteins consistently showed differential binding that was unrelated either to membrane permeability or to the possession of an X or Y chromosome. This indication of heterogeneity at the sperm surface, independent of the presence of a particular sex chromosome, emphasizes the need for caution in interpreting the results of antibody binding experiments. When an antibody consistently binds to about 50% of the sperm population, it is tempting to associate this with recognition of X or Y chromosome-bearing spermatozoa. However, only by checking antibody associations with sperm populations separated by FACS can such a link be demonstrated reliably. This is borne out by studies of one candidate immunological marker, the H-Y antigen, which is found exclusively in male mammalian tissues (Wachtel *et al.*, 1975). A number of researchers have shown that antibodies to H-Y bind only a proportion of the sperm population, and it has been postulated that these are spermatozoa carrying a Y chromosome. Attempts to prove this hypothesis have led to conflicting results; while some studies have indicated that treatment with antiserum to H-Y before fertilization biases the sex ratio of embryos (Bennett and Boyse, 1973; Zavos, 1983) or have suggested a correlation between anti-H-Y antibody binding and the presence of the Y chromosome (Ali *et al.*, 1990), others have found no evidence of a linkage between the two (Ohno

and Wachtel, 1978; Hoppe and Koo, 1984). The advent of the FACS sorting technique enabled Hendriksen *et al.* (1993) to carry out a study of the binding characteristics of seven anti-H-Y antibodies to bovine and porcine spermatozoa sorted by this technique and they concluded that, although antibodies did not bind to all spermatozoa, they showed no preferential association with Y chromosome-bearing spermatozoa.

Although a sex-specific surface antigen was not identified in the present study, this does not prove that such a marker is absent. The results do suggest, however, that if an antigen is present, it is a minor component of the membrane with a low antigenicity, and that an immunological approach to sperm sexing is unlikely to prove fruitful unless a gene product exclusive to one of the sex chromosomes can be identified and isolated. High resolution two-dimensional SDS-PAGE is one route by which such an antigen can be sought. Preliminary results indicate at least one sex-linked difference in protein expression of spermatozoa, and further work with this technique may identify other distinctions between X and Y chromosome-bearing spermatozoa. Another approach may be based on the recent finding that H-Y expression is linked to the SMC gene sited on the Y chromosome. Two regions showing substantial differences from the X chromosome homologue, SMCX, and coding for peptides unique to the Y chromosome have been identified (Scott *et al.*, 1995; Wang *et al.*, 1995). Such information should enable Y chromosome-specific peptide antibodies to be produced which may be capable of differentiating between types of spermatozoa, although current evidence indicates that H-Y antigens are unlikely to be associated with the cell surface.

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