Analysis of the murine sperm surface with monoclonal antibodies

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Summary. Hybridomas secreting monoclonal antibodies reactive with murine spermatozoa were produced by fusion of myeloma cells with spleen cells from C57BL/6J mice immunized with spermatozoa from mice of the same strain. All antisperm antibodies were of the μ (mu) immunoglobulin heavy chain class; only one (MS-1) bound *S. aureus* protein A. Antibody MS-1 recognized an antigen present on the sperm acrosomal cap, on the surface of cells from liver and kidney and from some cultured cell lines. The subunit molecular weight (69 000) of the polypeptide reactive with MS-1 was determined by SDS-PAGE analysis of sperm membrane proteins followed by their electrophoretic transfer to nitrocellulose.

Introduction

Knowledge of the molecular structure of the mammalian sperm surface is important for an understanding of the events of fertilization, the involvement of gametic antigens in infertility and for the evaluation of an immunological approach to contraception (Metz, 1972). The spermatozoa of mammals gain the ability to fertilize during passage through the epididymis (Bedford, Cooper & Calvin, 1972; Feuchter, Vernon & Eddy, 1981). Amongst the changes concomitant with the formation and maturation of the male gamete is the appearance of several differentiation antigens (Touillet & Voisin, 1974; Millette & Bellvé, 1977). Several studies have also documented the presence of histocompatibility antigens on spermatozoa (Fellous & Dausset, 1970; Goldberg, Aoki, Boyse & Bennett, 1970; Goldberg, Boyse, Bennett, Scheid & Carswell, 1971; Vojtiskova & Pokorna, 1972; Erickson, 1972) and it has been suggested that these may be important in cell-cell recognition at the gametic level (Festenstein, Halim & Arnaiz-Villena, 1979). The haploid expression of HLA antigens on human spermatozoa (Fellous & Dausset, 1970) and the limited expression of H-Y (male-specific) antigen on mouse spermatozoa (Bennett & Boyse, 1973) have promised exciting opportunities for manipulation of the sex ratio. A major disadvantage of conventional antisera employed in the serological investigation of sperm surface molecules is that they contain antibodies against multiple determinants, frequently rendering the interpretation of results ambiguous. The immortalization of single antibody-secreting cells by somatic cell hybridization (Köhler & Milstein, 1975) permits production of highly specific reagents (monoclonal antibodies) that recognize individual determinants. The aim of the present study was to investigate the mouse sperm surface using monoclonal antibodies produced after autosomal syngeneic immunization.

Materials and Methods

Immunization and cell fusion. Two 8-week-old female C57BL/6J (B6) mice were immunized by intraperitoneal (i.p.) injection of 5×10^6 spermatozoa in Dulbecco's phosphate-buffered saline pH 7.4 (PBS) from B6 males. They were boosted after 11 days with 5×10^6 spermatozoa i.p. and after

156 and 163 days with 10^7 spermatozoa intravenously. Spleens were removed 3 days later and used as a source of immune cells for fusion to the BALB/c myeloma line P3-NS/1-Ag-1 (obtained from Dr P. N. Goodfellow, ICRF, Lincoln's Inn Fields, London, U.K.) at a spleen cell to myeloma cell ratio of 5 to 1. Fusion products were suspended in HAT selection medium (RPMI 1640 containing 20% fetal calf serum, antibiotics, 10^{-4} M-hypoxanthine, 10^{-6} M-aminopterin, 1.6×10^{-5} M-thymidine, 1 mM-oxaloacetic acid, 0.45 mM-sodium pyruvate, 0.2 units porcine insulin/ml and 12.5 mM-3-(*N*-morpholine)propane sulphonic acid (MOPS); modified from Kennet, Denis, Tung & Klinman, 1978). The medium also contained 10^6 male BALB/c mouse macrophages/ml as feeder cells and was dispensed into twelve 96-well microtitre plates. Cultures were fed by replacing the medium in the wells every 3 or 4 days. Cloning was performed twice by limiting dilution.

Serological techniques. Supernatants from cultures showing growth were tested in an indirect cellular radioimmunoassay (Mason & Williams, 1980) against mouse spermatozoa. Duplicate aliquants (50 μ l) of culture supernatant were incubated at 4 °C for 60 min or overnight with 2×10^5 glutaraldehyde-fixed (Williams, 1973) target cells in 50 μ l PBS containing 2% bovine serum albumin (PBS-BSA). The target cells were washed three times and resuspended in 50 μ l column-purified rabbit F(ab')₂ antimouse immunoglobulin (a gift from Dr A. F. Williams, MRC Cellular Immunology Group, Oxford, U.K.) labelled with iodine-125 by the method of Jensenius & Williams (1974). The specific activity of the labelled antibody was approximately 60 μ Ci/ μ g; 2.5×10^5 c.p.m. were added to each well. After a further incubation at 4 °C for 60 min the cells were washed 3 times and transferred to clean tubes for counting. Background binding was assessed in wells containing target cells suspended in PBS-BSA. Binding three times above background was taken as a positive result. Selected supernatants were subsequently tested more extensively against panels of glutaraldehyde-fixed target cells (Text-fig. 1).

Protein A binding. Antisperm antibodies were examined for protein A binding in parallel cellular radioimmunoassays with mouse spermatozoa by comparing ^{125} I-labelled rabbit F(ab')₂ antimouse immunoglobulin and ^{125} I-labelled protein A (Amersham International, Amersham, U.K.) as detecting reagents. Biosynthetically labelled monoclonal antibodies were then examined for direct binding to *Staphylococcus aureus*. Hybridoma cells (2×10^6) were washed 3 times in PBS and incubated overnight (16 h) in 1 ml Ham's F10 medium, lacking lysine but supplemented with 10% dialysed FCS, 12.5 mM-MOPS and 12.5 μ Ci L-[^{14} C]lysine (sp. act. 339 mCi/mmol; Amersham International)/ml. Heat- and formalin-fixed *S. aureus* (Cowan I strain, NCTC-8530, CAMR Microbial Products, Porton Down, Wilts, U.K.) were washed three times with PBS containing 0.5% Nonidet P40 (NP40; Shell, Manchester, U.K.) and 25 μ l of a 10% (v/v) bacterial suspension incubated with 10^5 trichloroacetic acid-precipitable c.p.m. ^{14} C-labelled monoclonal antibody for 30 min at room temperature (21 °C). After washing the bacteria 3 times with PBS-NP40, bound radioactivity was eluted by boiling the cells for 2 min in 25 μ l 0.0625 M-Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecyl sulphate (SDS) and 0.001% bromophenol blue, and electrophoresed on 10% polyacrylamide gels as described by Laemmli (1970). The gels were impregnated with autoradiography enhancer (En³Hance: New England Nuclear, Southampton, U.K.), dried and exposed to X-ray film (Kodak Xomat RP: Kodak, Glasgow, U.K.) at -70 °C. Apparent molecular weights were calculated according to the method of Weber & Osborn (1969).

Determination of immunoglobulin class. The class of the secreted monoclonal antibody was determined by radial immunodiffusion using heavy chain (Miles Laboratories, Slough, U.K.) and light chain (Nordic Immunological Laboratories, Maidenhead, U.K.) class specific antisera.

Immunofluorescence and FACS analysis. Fresh B6 spermatozoa washed 3 times in PBS were resuspended in an excess of culture supernatant and incubated at 4 °C for 15 min. The cells were washed 3 times and resuspended in fluorescein isothiocyanate-conjugated rabbit antimouse immunoglobulins (Nordic Immunological Laboratories) diluted 1 in 5 in PBS for a further 15 min at 4 °C. After washing 3 times, the cells were examined and photographed using a combination of phase-contrast and u.v. epi-illumination in a Leitz Ortholux II microscope. Additional samples were examined and sorted in a fluorescence-activated cell sorter (FACS) (Green, Molloy & Steel, 1980).

Biochemical analysis. Spermatozoa from the vasa deferentia and epididymides of 20 BALB/c mice were washed 3 times in PBS and membrane proteins were extracted by resuspending the spermatozoa in 0.5 ml extraction buffer (0.5% w/v NP40 in 10 mM-Tris-HCl pH 7.2, 0.15 M-NaCl, 0.02% w/v NaN_3 , 0.1 mM-phenylmethylsulphonyl fluoride 1 mM- MgCl_2 , 500 kallikrein inactivator units/ml aprotinin (Trasylol: Bayer, Haywards Heath, Sussex, U.K.) for 20 min on ice. Insoluble material was removed by centrifugation for 15 min at 4 °C in an Eppendorf model 5414 Microfuge. Aliquants of the supernatant were electrophoresed in 5–20% linear concentration gradient SDS-polyacrylamide gels (Laemmli, 1970) under reducing and non-reducing conditions. The separated proteins were transferred electrophoretically from the gel onto a sheet of nitrocellulose (Schleicher and Schuell BA85: Anderman & Co., Surrey, U.K.) according to the method of Burnette (1981).

Immediately after transfer the nitrocellulose sheet was immersed in a solution containing 10 mM-Tris-HCl pH 7.4, 0.15 M-NaCl and 2.5% w/v bovine serum albumin (Tris-saline-BSA) for 30 min at room temperature and then incubated in hybridoma culture supernatant for 90 min. The sheet was washed for 10 min in Tris-saline, two changes of Tris-saline containing 0.05% w/v NP40, each for 20 min, and finally for 10 min in Tris-saline alone. The nitrocellulose was then

Text-fig. 1. Binding of antibody MS-1 to spermatozoa, adult mouse tissues and cultured cell lines.

incubated for 30 min with Tris-saline-BSA containing 10^5 c.p.m. ^{125}I -rabbit $\text{F}(\text{ab}')_2$ antimouse immunoglobulin/ml, washed as described above, dried at 37°C and exposed at -70°C to Kodak X-Omat RP X-ray film.

Results

Of the 1056 cultures (one plate was lost through contamination), 501 (47%) showed growth and 24 (2%) produced antibody recognizing the immunogen by radioimmunoassay. The specificity of these antibodies was further studied on a diverse panel of spermatozoa, adult mouse tissues and cultured cell lines; none was able to discriminate between male and female cells. The results for the culture (MS-1) showing the most restricted tissue distribution are shown in Text-fig. 1. The monoclonal antibody MS-1 appeared to react with an antigen present on mouse spermatozoa, liver and kidney which is absent from other somatic tissues of the mouse. The antigen was also present to a variable degree on some of the cultured cell lines examined.

All 24 of the antisperm antibodies were of the mu (μ) and kappa (κ) heavy and light chain classes of immunoglobulin by their reactivity in radial immunodiffusion with antisera specific for those classes and by their failure to react with antisera directed against other classes and subclasses of mouse immunoglobulins. Of 14 of these mouse IgM monoclonal immunoglobulins examined,

Text-fig. 2. SDS-PAGE analysis of biosynthetically labelled and reduced monoclonal antibodies bound to and eluted from *S. aureus*. Of 4 antisperm antibodies (lanes H-K) only MS-1 (lane I) was reactive. Elution of an IgG antibody (lane D) from earlier work is shown for comparison. Lanes A and M contained ^{14}C -methylated protein standards: myosin (M_r 200 000), phosphorylase b (M_r 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), carbonic anhydrase (M_r 30 000), and ribonuclease A (M_r 14 300).

Text-fig. 3. Acrosomal staining of spermatozoon with antibody MS-1: (a) phase contrast; (b) immunofluorescence.

only MS-1 bound ^{125}I -labelled protein A, as shown by radioimmunoassay. This was subsequently confirmed by SDS-PAGE analysis of biosynthetically labelled monoclonal antibodies bound directly to and eluted from *S. aureus* (Text-fig. 2). The apparent molecular weight (78 000) of the heavy chain of antibody MS-1 is consistent with values previously reported for IgM macroglobulins (Cohen & Milstein, 1967; Metzger, 1970).

When fresh spermatozoa were examined for reactivity with MS-1 by indirect immunofluorescence the fluorescent label was confined to a crescent-shaped area of the anterior acrosomal region of the sperm head (Text-fig. 3); 42% (range 18–59%) of spermatozoa showed acrosomal staining with MS-1 under the conditions described. None of the contaminating cells were stained.

Text-fig. 4. Isometric plot of fluorescence intensity against scatter (representing cell size) after FACS analysis of unfractionated spermatozoa stained with antibody MS-1 and FITC-conjugated rabbit antimouse immunoglobulins.

Text-fig. 5. SDS-PAGE analysis of sperm membrane proteins after transfer to cellulose nitrate. Reduced (lane A) and non-reduced (lane C) samples were electrophoresed on the same gel. ^{14}C -methylated protein standards (lane B) were myosin (M_r 200 000), phosphorylase b (M_r 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), carbonic anhydrase (M_r 300 000) and ribonuclease A (M_r 14 300).

Sperm acrosome staining was not seen when the other antisperm monoclonal antibodies were substituted for MS-1 or when the conjugated antiserum was used alone. An isometric plot of relative light scatter of individual cells (representing cell size) and fluorescent intensity for an unfractionated sample of vas deferens and epididymal spermatozoa analysed with a flow cytometer is presented in Text-fig. 4. The fluorescence pattern of MS-1 on spermatozoa is bimodal and an unstained mixed population of spermatozoa and contaminating cells is also detected. It was possible to sort spermatozoa into fluorescence-positive and fluorescence-negative populations.

The molecular weight of the antigen recognized by antibody MS-1 was investigated by SDS-PAGE analysis of sperm membrane proteins and by their electrophoretic transfer to nitrocellulose and development with monoclonal antibodies and ^{125}I -labelled rabbit antimouse immunoglobulin. MS-1 bound predominantly to a protein with an apparent molecular weight of 69 000 after pre-treatment of NP40-solubilized proteins with the sulphhydryl reagent 2-mercaptoethanol (reducing conditions). Under non-reducing conditions the antibody reacted with several protein species with apparent molecular weights greater than 98 000 (Text-fig. 5).

Discussion

The percentage of hybrid cell lines generating antisperm antibodies was low (2%), in close agreement with values reported by Feuchter *et al.* (1981) following isogenic immunization of B6 male mice with spermatozoa.

By virtue of their immunologically privileged environment it has been suggested that when spermatogenic cells are used for syngeneic immunization, they should elicit a tissue-specific response (Bechtol, Brown & Kennet, 1979; Bechtol, Jonak & Kennet, 1980; Feuchter *et al.*, 1981). In the present study autosomal syngeneic immunizations were used because spermatozoa are equally foreign to the immune system of an isogenic virgin female and in addition may elicit an allergic response directed against gene products of the Y chromosome. The concentration of H-Y antigen on spermatozoa is considerably higher than on male spleen cells (Katsh, Talmage & Katsh, 1964). Spermatozoa are amongst the few cell types susceptible to killing by anti-H-Y antisera and complement (Hall & Wachtel, 1980); that spermatozoa have a higher density of H-Y antigen than other cells is implicit in this observation (Winn, 1962; Linscott, 1970; Johnson, 1970). It would therefore seem that immunization and assay with syngeneic spermatozoa is an appropriate system for the selection of Y chromosome-limited and sperm-specific monoclonal antibodies. It is clear from the present data, however, that none of the antibodies obtained possessed anti-H-Y activity since they did not discriminate between male and female cells. It has been reported that H-Y antigen is limited in distribution to the region of the acrosomal cap in mouse spermatozoa (Koo, Stackpole, Boyse, Hämmerling & Lardis, 1973). The antigen recognized by antibody MS-1 is also confined to the acrosomal region of the sperm head but was not detected on adult male spleen, epidermis or thymus tissues which are H-Y positive in transplantation and serological assays (Gasser & Silvers, 1972; Wachtel, 1977).

Mouse spermatozoa have been shown to bind immunoglobulins specifically via the Fc portion of the molecule. It is unlikely, however, that the binding of the MS-1 antibody occurs by this mechanism since the sperm receptors are Fc γ -specific (Sethi & Brandis, 1980), and, in addition, none of the other IgM monoclonal antibodies examined produced acrosomal staining.

The finding that all the sperm-reactive monoclonal antibodies produced after autosomal syngeneic immunization were of the IgM class was unexpected. There was no obvious selection for immunoglobulins of this class since polyvalent rabbit anti-mouse immunoglobulin was employed as detecting reagent in the serological screening procedures. Class restriction of the humoral response may be influenced by the nature of the immunogen or result from a genetic predisposition for the selective production of immunoglobulin of defined isotype. Evidence supporting the latter possibility has been obtained in studies of B6 mice which produce predominantly IgM antibodies after hyper-immunization with sheep red blood cells (Silver & Winn, 1973; Haber & Winn, 1981). The observation that of the 14 B6 monoclonal IgM immunoglobulins only MS-1 bound protein A is consistent with a previous report (MacKenzie, Gutman & Warner, 1978) that murine protein A-reactive IgM proteins are uncommon. It would be inappropriate, therefore, to use this reagent in preliminary screening assays for monoclonal antibodies under conditions favouring selection for IgM.

The identification of a specific antigen on spermatozoa is confounded by the presence of auto-antibodies in normal and conventional immune sera and by the observation that only a small fraction of the cells are reactive in serological assays (Fellous, Gachelin, Buc-Caron, Dubois & Jacob, 1974). The application of homogeneous antibodies produced *in vitro* by hybridoma technology after appropriate immunization and screening will largely circumvent the first of these problems. In addition it is unnecessary (and futile) to perform adsorption studies (as shown by H-Y serology, Beutler, Nagai, Ohno, Klein & Shapiro, 1978; Koo, 1981) which are often cumbersome and generally result in loss of titre (Yelton & Scharff, 1981). The ability to examine cross-reactions, such as those observed in the present study, is one of the most powerful applications of monoclonal antibodies (Yelton & Scharff, 1981).

Biochemical analysis showed that the MS-1 antibody bound predominantly to a sperm membrane protein of apparent molecular weight 69 000 (lane A, Text-fig. 5) but binding also occurred to other higher molecular weight proteins. Further studies are required to determine whether this indicates that identical epitopes, possibly carbohydrate in nature, are present on these higher molecular weight proteins or that the higher molecular weight components represent

aggregates or polymers of the 69 000 M_r protein. The alternative explanation that the MS-1 antibody is polyclonal seems unlikely since the hybridoma has been subcloned twice and grown continuously *in vitro* for several months.

Suspensions of mouse spermatozoa have been shown to behave as heterogeneous populations in cytotoxicity assays (Yanagisawa, Bennett, Boyse, Dunn & Dimeo, 1974) although no plausible explanation has been offered to account for such results (Fellous *et al.*, 1974). Similarly, MS-1 antigen was detectable on only 42% of spermatozoa in immunofluorescence studies. This heterogeneity is probably due to the mixed populations of mouse spermatozoa, derived from the vasa deferentia and the epididymides, commonly employed in serological investigations. Biochemical data indicate that the MS-1 antigen is a disulphide-bonded polymeric protein of high molecular weight. A high content of cysteine is present in spermatozoa from which disulphide cross links are formed during their passage through the epididymis (Calvin, 1975). It is possible, therefore, that the immunofluorescent-negative spermatozoa observed in the present study represent less mature cells.

The topographically restricted distribution of the MS-1 antigen is consistent with the mosaic distribution of sperm-surface components demonstrated by a variety of cell surface labelling procedures (Koehler, 1978) and more recently with monoclonal antibodies (Myles, Primakoff & Bellvé, 1981; Feuchter *et al.*, 1981). It should now be possible to examine the relationship between such molecules and specific functions of the sperm surface, for example the involvement of the MS-1 protein in the acrosome reaction and its influence on sperm function during fertilization.

Postmeiotic expression of sperm antigens has been extensively sought with few convincing results (Johnson & Howe, 1975) and it remains to be shown that continued haploid gene activity actually occurs (Festenstein *et al.*, 1979). The demonstration here that it is possible to sort sperm populations in a flow cytometer after suitable immunofluorescent staining and that cells can be recovered in a viable state (Arndt-Jovin & Jovin, 1977; Green *et al.*, 1980) makes it feasible to test this hypothesis directly using artificial insemination when suitable probes are available.

We thank Dr V. van Heyningen for helpful advice; Dr C. M. Steel for critically reading the manuscript; Professor Sir Michael Woodruff, Dr M. Hooper and Dr R. A. J. McIlhinney for cultured cell lines; Dr D. K. Green for the FACS analysis; Mr N. Davidson and Mr D. Stuart for photographic illustrations; Mrs I. Cunnison for secretarial assistance and the staff of the Animal House, Western General Hospital for supplying the animals.

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