J. Embryol. exp. Morph. **93**, 197–211 (1986)
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Biochemical characterization of the adhesion-related differentiation antigen XT-1

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SUMMARY

The XT-1-molecule, an adhesion-related differentiation antigen of male mouse germ cells, is a 34 000 M_r glycoprotein with major charge isomer at pI 5·1 and is an integral component of the cell membrane. On large late pachytene spermatocyte, the molecule is present at a concentration of $2{\cdot}5\times10^3$ molecules μm^{-2}, which approximates HLA/ABC concentration on lymphocytes. By comparing the reactivity of four anti-XT-1 monoclonal antibodies, three of which elicit germ cell–germ cell adhesion, we have defined two distinct surface regions of the XT-1-molecule. The relationship of the XT-1-molecule with other known adhesion-related molecules and testicular antigens is discussed.

INTRODUCTION

The cell membrane has traditionally defined the physical limits of the cell. In contemporary thought, the delimiting concept is expanded to the cell 'surface' including both integral and peripherally associated components and junctional complexes with other cells. The molecular composition of the surface is presumed to reflect the functional requirements of the particular cell type (e.g. immunoglobulin molecules on the surface of B lymphocytes). One approach to understanding the multiple functions of the cell surface has been the identification of components unique to a particular cell type. The most extensive use of this approach has been through study of 'differentiation antigens' (Boyse & Old, 1969).

Several populations of cell surface differentiation antigens of testicular germ cells have been described using polyclonal immune sera, and recently monoclonal antibodies have been used to identify individual antigens. O'Rand has characterized the rabbit sperm antigen RSA-1 (O'Rand & Porter, 1979, 1982); Gaunt (1982) has characterized the mouse germ cell antigen 1B3; Fenderson *et al.* (1984) have identified several carbohydrate determinants; and Lingwood & Schachter

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Key words: cell–cell adhesion, monoclonal antibodies, germ cells.

(1981) have identified a glycolipid differentiation antigen. Many others have identified germ cell surface molecules (O'Rand & Romrell, 1977, 1980; Millette & Bellvé, 1977, 1980; P. S. Tung & Fritz, 1978; K. S. K. Tung *et al.* 1979), but these have not yet been characterized.

An alternative method to identify cell surface molecules is through analysis of quantitative and qualitative shifts in cell surface composition of germ cells during spermatogenesis. This involves the comparison of electrophoretic gel patterns of proteins and glycoproteins solubilized from germ cells that have been separated according to size (i.e. developmental stage) (Boitani *et al.* 1980; Millette & Moulding, 1981; Romrell *et al.* 1982; Kramer & Erickson, 1982; Stern *et al.* 1983; O'Brien & Millette, 1984). A major disadvantage of this approach is that it is dependent on producing a single-cell suspension by proteolytic harvesting, which may destroy or irreversibly modify protease-sensitive molecules.

Using monoclonal antibody XT-I, we have identified a protease-sensitive differentiation antigen (XT-1-antigen) of mouse germ cells (Bechtol *et al.* 1979). By quantitative absorption, the antigen is testis-specific (i.e. not found on spleen, liver, kidney, brain or whole ovary; Bechtol *et al.* 1979). Immunoperoxidase staining of cells mechanically dissociated from the testis shows surface staining of many of the germ cells (Bechtol *et al.* 1980). Immunohistochemical analysis of sections from juvenile and adult mouse testis show that XT-1-antigen becomes detectable on early, leptotene/zygotene spermatocytes and increases during the long pachytene spermatocyte phase. As the haploid cell develops, the distribution of the XT-1-determinant shifts from its relatively uniform surface distribution on spermatocytes to a more restricted localization on the extreme base of the head, tail and cytoplasmic lobe of the elongating spermatid (Bechtol, 1984). XT-1-antigen is not detected on juvenile Sertoli cells in normal testis, on Sertoli cells in aspermatogenic segments of normal adult testis, or on Sertoli cells in genetically germ-cell-depleted (W^x/W^v) adult mice (Bechtol, 1984). Thus, detection of the antigen in the testis is dependent on the presence of germ cells of the appropriate developmental stage.

We have recently shown that each of three monoclonal antibodies (XT-I, MT-23, MT-24), upon binding to the XT-1-molecule, elicits germ cell–germ cell adhesion, while a fourth monoclonal antibody (MT-29) fails to elicit this adhesion; adhesion is also produced by binding of the monovalent Fab fragment of XT-I-antibody (Ho & Bechtol, 1985). Here we report the biochemical characterization of the XT-1-differentiation-antigen and discuss its relationship with other known adhesion-related molecules and testicular antigens.

MATERIALS AND METHODS

Radiolabelling of cells, antigen solubilization and immunoprecipitation

Testis cells were prepared by mechanical disruption of adult strain 129 mouse testis as previously described (Bechtol *et al.* 1979). These cells were radioiodinated by the lactoperoxidase/glucose oxidase method (Marchalonis *et al.* 1971) using 0·5 mCi ^{125}I (Amersham) for 10^7 cells. Cells were biosynthetically labelled with [^{35}S]methionine by incubation of 1 cm

segments of testis tubule in 0·2 ml methionine-free minimum essential medium (Gibco) plus 5 % dialysed foetal calf serum (KC Biological) and 80 μCi [^{35}S]methionine (1·2 mCi mmol^{-1}, Amersham). Cells from at least 14 such segments were pooled and aliquots of the pool used for immunoprecipitation with different antibodies. Alternatively, cells from 17 days *post partum* (*p.p.*) mice were placed in outgrowth culture (Ho & Bechtol, 1985) for 24 h, followed by [^{35}S]methionine (400 μCi ml^{-1}) labelling overnight. In all cases, approximately 10^7 washed cells were solubilized in 1 ml of 0·5 % NP40 in NETP [N = 150 mM-NaCl; E = 5 mM-ethylene diamine tetraacetic acid; T = 50 mM-Tris/HCl buffer, pH 7·4; P = 2 mM-freshly prepared phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor] and the supernatant was precleared by incubation once on ice with excess *Staphylococcus aureus* Cowan I (Calbiochem) and once with *S. aureus* coated with rabbit IgG anti-mouse Ig. 100 μl of precleared supernatant was mixed with 200 μl of monoclonal antibody in serum-free culture supernatant plus 10 μl of washed packed *S. aureus*. If monoclonal antibodies that do not bind protein A (i.e. the MT monoclonals) were used, then the *S. aureus* was precoated with 0·1 mg of affinity-purified rabbit anti-rat Ig (Zymed) for use in immunoprecipitation. After incubation overnight at 4 °C, the *S. aureus* was washed twice with 0·05 % NP40 in NET (modified NETP, see above), and the precipitate eluted for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with 40 μl of Laemmli (1970) sample buffer with or without 5 % β-mercaptoethanol or for isoelectric focusing as described by O'Farrell (1975).

The requirements for XT-1-antigen solubilization from testis cells were tested by treating aliquots of radioiodinated cells with 3 M-KSCN, 1 M-urea or 0·5 % NP40, each in balanced buffered salt solution (BBSS), or with distilled water or BBSS alone for 15 min on ice. After centrifugation for 4 s in a Beckman microfuge (Model B), the supernatants were dialysed against BBSS, immunoprecipitated with XT-I-antibody and run on SDS–PAGE. Only the NP40 extract showed a large specific immunoprecipitate on autoradiography. Alternatively, unlabelled cells were similarly incubated, then washed in BBSS and used as targets in a radioimmunoassay for binding of several dilutions of XT-I-antibody as previously described (Bechtol *et al.* 1979). Only the NP40-treated cells showed extensive depletion of binding sites per tube.

SDS–PAGE, NEPHGE gels, autoradiography, silver staining and electroblot

SDS–PAGE was performed according to the procedure of Laemmli (1970). NEPHGE gels were run according to the method of O'Farrell *et al.* (1977) using pH 3·5 to 10 ampholines (LKB). Autoradiography of Coomassie blue-stained and dried gels was accomplished at −70 °C using XAR-5 film (Kodak) for ^{125}I and RX film (Fuji) for ^{35}S. Silver staining was performed by washing the gel in 25 % isopropanol and 10 % acetic acid for 1 h, in 10 % isopropanol for 1 h, in 10 % buffered glutaraldehyde for 1 h, and in 500 ml of deionized water overnight. The gel was then stained as described by Oakley *et al.* (1980). For electroblotting, the NP40 extract of testis cells was separated by SDS–PAGE in a 7 % gel, transferred to nitrocellulose using a Biorad transblot unit, incubated with the monoclonal antibodies as described by Towbin *et al.* (1979) and Burnette (1981), incubated with ^{125}I-rabbit anti-mouse Ig, washed, dried and analysed by autoradiography using XAR-5 film (Kodak). ^{14}C-labelled molecular weight markers were from BRL.

Lens culinaris *lectin affinity column*

The *Lens culinaris* lectin column was prepared as described in Knudsen *et al.* (1981). 10^7 radioiodinated cells were lysed in 1 ml of buffer B (0·5 % NP40 in NT with 0·5 mM-$CaCl_2$) with PMSF. The supernatant was applied to a 2·0 ml *Lens* column that had been preeluted with 5 ml of 10 % glucose in buffer B and washed with 5 ml of buffer B. Lysate was allowed to bind for 10 min at 4 °C, then 0·5 ml fractions were collected as the unbound lysate was washed through with 25 ml of buffer B. 2 ml of 10 % glucose in buffer B were run onto the beads and incubated overnight at 4 °C. The next morning the column was washed with 10 ml of 10 % glucose in buffer B, and 0·5 ml fractions were collected. For the unbound lysate and each of the elutions, 100 μl of the 0·5 ml fraction containing the most counts per minute was immunoprecipitated, run on 10 % SDS–PAGE and autoradiographed.

Antibodies and hybridoma production

XT-I (IgG_{2a}) is a mouse monoclonal antibody produced as previously described (Bechtol *et al.* 1979). The subclone of XT-I presently in use is a derivative which has lost the $\gamma 1$ heavy chain (Bechtol, 1984) and κ light chain of the P3-X63Ag8 myeloma parent, as determined by Ouchterlony double diffusion with anti-class reagents (Bionetics) and by SDS–PAGE. MT-23 (IgG_{2a}) MT-24 (IgG_{2b}) and MT-29 (IgG_{2a}) rat monoclonal antibodies were produced by immunization with substantially enriched XT-1-antigen. The dialysed KSCN-eluted antigen from XT-I-antibody beads (see Results) was emulsified in complete Freund's adjuvant (CFA) injected into four subcutaneous sites in a female Lewis rat. 0·4 ml of an identical antigen preparation in NET was injected intraperitoneally 21 days later. After the second immunization, the serum of this rat contained significant binding activity to mouse testis cells over that shown by its preimmune serum. The rat was immunized intravenously with two 0·2 ml aliquots of a third XT-1-antigen preparation on days 54 and 56, and the immune splenic lymphocytes were fused with the SP2/0 mouse myeloma line on day 57. Each of the three antigen preparations was obtained from 10^7 cells from testes of adult strain 129 mice. Positive clones were detected by testing the supernatants of growing hybrid wells by radioimmunoassay using a suspension of mouse testis cells as target and affinity-purified rabbit IgG anti-rat IgG (H & L) (Kirkegaard & Perry Laboratories, Inc.) radioiodinated by the chloramine T method (Klinman & Howard, 1980). Several wells produced antibodies that bound to testis cells, and they were subcloned by limiting dilution. The clonal nature of each line was confirmed by the presence of a single Ig heavy and light chain in silver-stained SDS–PAGE of supernatants grown in serum-free medium. Anti-rat antibody class reagents were from Bethyl Laboratories Inc. UK8 is an IgG_{2a} mouse monoclonal antibody produced by fusion of SP2/0 (Shulman *et al.* 1978) myeloma cells with splenic lymphocytes from a C3H.SW female mouse. HS-19 is an IgG_1 mouse monoclonal antibody that reacts with the acrosome (Florman *et al.* 1984). HB-11 is an IgG_{2a} mouse monoclonal antibody binding $H\text{-}2K^bD^b,K^d$ (Ozato & Sachs, 1981; antibody 20-8-4S, obtained from American Type Culture Collection as HB-11). Fusions were performed as previously described (Bechtol *et al.* 1979). All hybridomas were grown in serum-free medium (Murakami *et al.* 1982). Affinity-purified rabbit anti-mouse Ig and anti-rat Ig (Kirkegaard & Perry Laboratories, Inc.) were radioiodinated by the chloramine T method. Immunoperoxidase staining was performed as previously described (Bechtol, 1984).

Monoclonal antibody affinity columns

To prepare monoclonal antibody affinity columns, XT-I-antibody was purified by binding 200 ml of serum-free culture supernatant to a 1·5 ml protein-A column (Pharmacia). Antibody was eluted at pH 4·5 (Ey *et al.* 1978), neutralized to pH 7·0, then dialysed against coupling buffer (0·5 M-NaCl, 0·1 M-$NaHCO_3$, pH 8·3). HS-19 serum-free supernatant was passed over a 10 ml anti-human transferrin (Cappel)-affinity column, and the unbound effluent was dialysed against coupling buffer. 4·5 mg of antibody was coupled to 1·5 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia) as described by Pharmacia. Greater than 90% of the applied antibody was bound to the Sepharose. For binding to antibody-coupled affinity beads, radioiodinated or unlabelled antigen was solubilized in 0·5% NP40 in NETP and bound to the antibody-derivatized beads overnight at 4°C. Unbound molecules were removed by washing with 0·5% NP40 in NET. Bound antigen was eluted by incubation in 3 M-KSCN in NET 15 min at 20°C and was dialysed *versus* NET to remove KSCN. In some cases the dialysed, eluted antigen was bound again to XT-I-antibody beads for 2 h at 4°C, then eluted with 3 M-KSCN and dialysed. The purity of eluates was assessed in SDS–PAGE followed by autoradiography or silver staining.

Radioimmunoassays and Scatchard-type analysis

Indirect radioimmunoassays were performed as described by Bechtol *et al.* (1979). For inhibition of binding, monoclonal antibodies were labelled by overnight incorporation of [^{35}S]methionine by the antibody-secreting hybridomas as described above for testis. Antibody in the medium was separated from unincorporated label by passage over a 10 ml G25 column and collection of the excluded volume. Binding of serial dilutions of the labelled antibodies to a

suspension of testis cells was assayed and an appropriate dilution of labelled antibodies chosen for inhibition studies. To test inhibition of binding, 25 μl of dilutions of serum-free supernatant containing unlabelled monoclonal antibodies or 25 μl of 0·5 % bovine serum albumin (BSA) were incubated with 50 μl of a suspension of testis cells in 0·5 % BSA in BBSS for 1 h on ice, then 25 μl of radiolabelled antibody were added and the incubation continued an additional hour. Cells were washed twice and the bound label counted. For quantitative, Scatchard-type binding assays, protein A purified XT-I-antibody of known concentration was enzymatically labelled with [^{3}H]*N*-hydroxylsuccinimydal proprionate (Ross *et al.* 1983). Germ cells were prepared from either 20-day-old mice or adult mice (greater than 2 months old) by mechanical disruption of the testis, while sperm were isolated from the epididymis of adult mice (Bechtol *et al.* 1979). Binding experiments were performed and analysed as described by Trucco *et al.* (1980) and Trucco & de Petris (1981), or by inhibiting the binding of aliquots of labelled antibody with various amounts of cold antibody (from 40 times the labelled amount of antibody to 1/10th the labelled amount). The calculated mean number of antigen sites per cell in the adult testis represents a minimum estimate of the mean antigen concentration on antigen-positive cells because a small number of antigen-negative cells (e.g. some Leydig cells and Sertoli cell fragments) was included in the cell sample.

RESULTS

XT-1 is an abundant cell surface differentiation antigen

Immunostaining of live and of fixed and sectioned cells demonstrated XT-1-antigen to be a cell-surface antigen (Bechtol *et al.* 1980; Bechtol, 1984). Scatchard-type binding experiments on testis cells revealed an average of 7×10^5 XT-1-molecules per cell in a cell suspension derived from adult testis by mechanical dissociation (Fig. 1), whereas testis cells from 20-day-old mice,

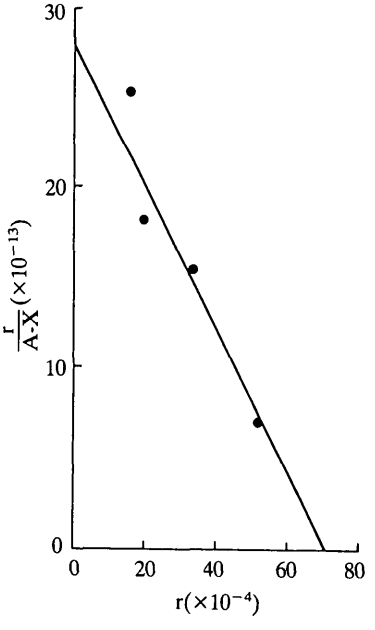

Fig. 1. Scatchard plot for binding of XT-I-antibody to suspension of cells from adult testis. XT-I-antibody was purified on a protein A affinity column and labelled with [^{3}H]*N*-hydroxysuccinimydal propionate.

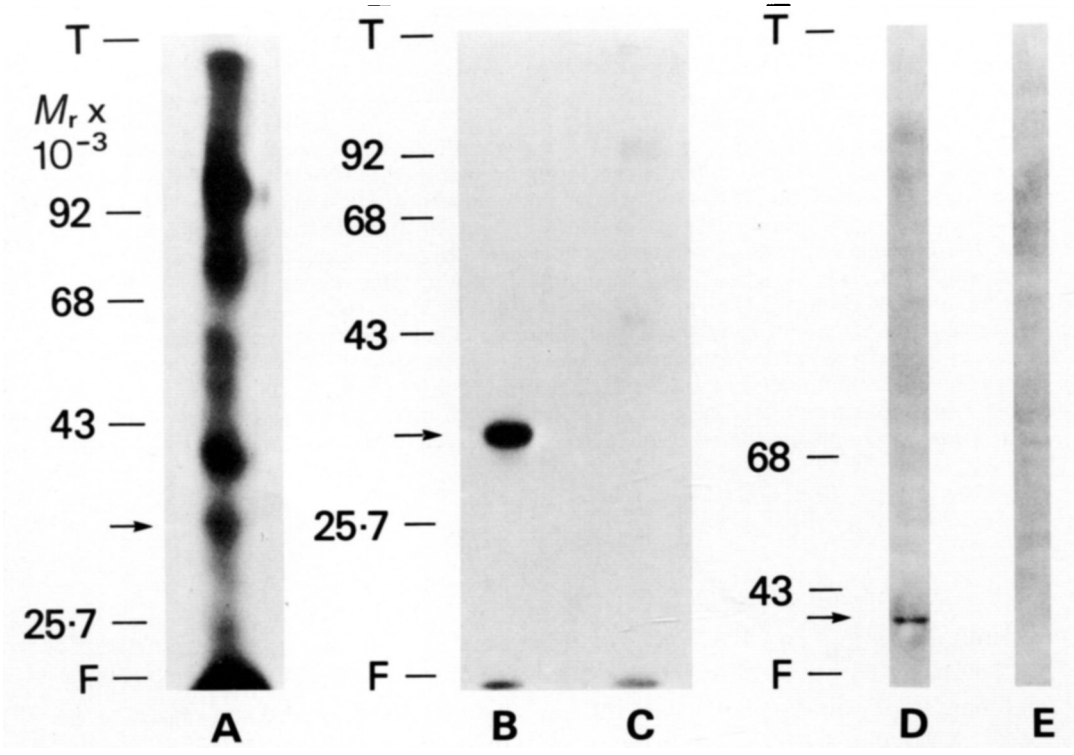

Fig. 2. Immunoprecipitation and Western transblot of XT-1-antigen. For immunoprecipitation (lanes B and C), a mechanically prepared suspension of cells from adult mouse testis was radioiodinated and solubilized in 0·5 % NP40. The total iodinated preparation is shown in lane A. Aliquots were immunoprecipitated with monoclonal antibodies XT-I-antibody (lane B) or HB-11 (lane C) and analysed on 10 % SDS–PAGE in the presence of 2-mercaptoethanol. Identical results are seen in nonreducing gels (data not shown). For Western transblot (lanes D and E), unlabelled cells were solubilized, run on 7 % SDS–PAGE, and transferred onto nitrocellulose by electrophoresis. Strips representing the different lanes of the gel were incubated with XT-I-antibody (lane D) or UK8 (lane E), followed by radioiodinated anti-mouse Ig. Arrow indicates XT-1-antigen. Bars mark from top to bottom: top of the running gel (T), molecular weight markers phosphorylase B (M_r 92 000), BSA (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 700) and the running front of the gel (F).

containing the maximum percentage of late pachytene spermatocytes, have an average of 2×10^6 XT-1-molecules per cell, and epididymal sperm have but 4×10^4 XT-1-molecules per cell (data not shown). These results are in accord with the antigen's distribution detected by absorption studies using mice of various juvenile and adult ages (Bechtol *et al.* 1979) and with the immunoperoxidase staining pattern of XT-I-antibody in testis tissue sections (Bechtol, 1984).

Immunoprecipitation of XT-1-antigen

Analysis of the XT-I immunoprecipitate of cell-surface-labelled adult testis cells in SDS–PAGE, in the presence of 2-mercaptoethanol as reducing agent, reveals a single major band at 34 000 M_r (Fig. 2B); an identical band is seen when

the immunoprecipitate is electrophoresed without reduction. That this immunoprecipitated band is the antigenic molecule is confirmed by electrophoresis in SDS–PAGE of NP40 extracts of testis cells, followed by electroblot transfer to nitrocellulose and radioimmunodetection. The major band detected with XT-I-antibody migrates at an M_r similar to that of the radiolabelled immunoprecipitated molecule (Fig. 2D).

Affinity purification of XT-1-molecule

A method for preparative isolation of the XT-1-molecule was devised involving NP40 solubilization of cells and specific binding to and elution from Sepharose

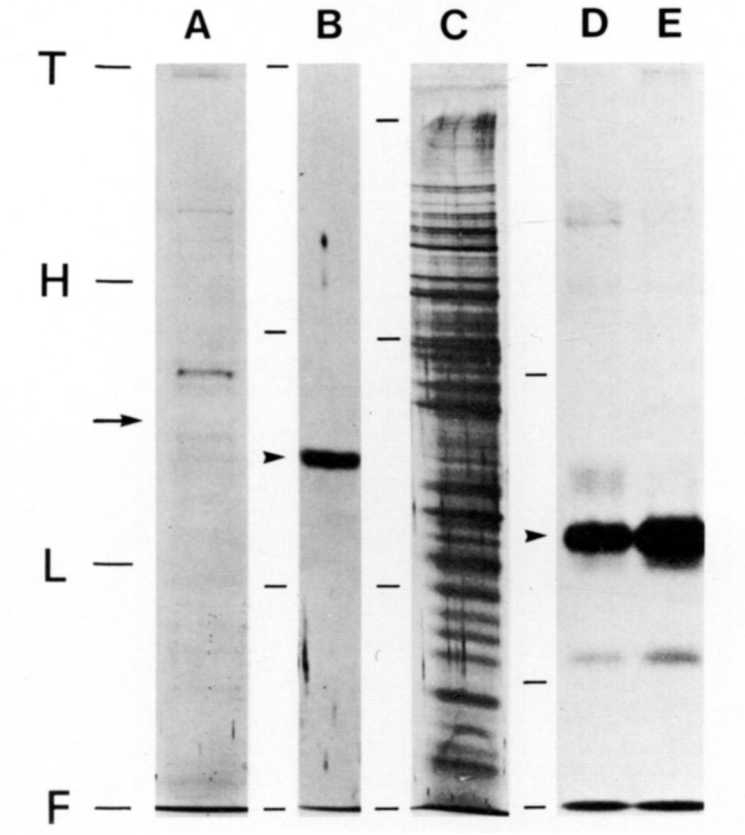

Fig. 3. Binding of XT-1-antigen to antibody affinity columns. Unlabelled NP40 cell extract was bound and eluted from HS-19-derivatized beads (A) or XT-I-derivatized beads (B), run in SDS–PAGE and silver stained. Lane C contains 1/500th the amount of total lysate used to produce the XT-1-antigen in lane B. Antigen localization in lane B was confirmed by autoradiography of labelled antigen run in the same gel. The NP40 extract of surface radioiodinated cells was bound to XT-I-antibody derivatized beads, eluted with KSCN and dialysed. The autoradiogram of 25 μl of this eluate is shown in lane D, while 100 μl of the same eluate was immunoprecipitated with XT-I-antibody (lane E). Arrows indicate XT-1-antigen. Bars indicate T, top of the separating gel; H, XT-I Ig heavy chain; L, XT-I Ig light chain; F, the running front of the gel. All lanes are from 10 % acrylamide gels except B, which was 12 %.

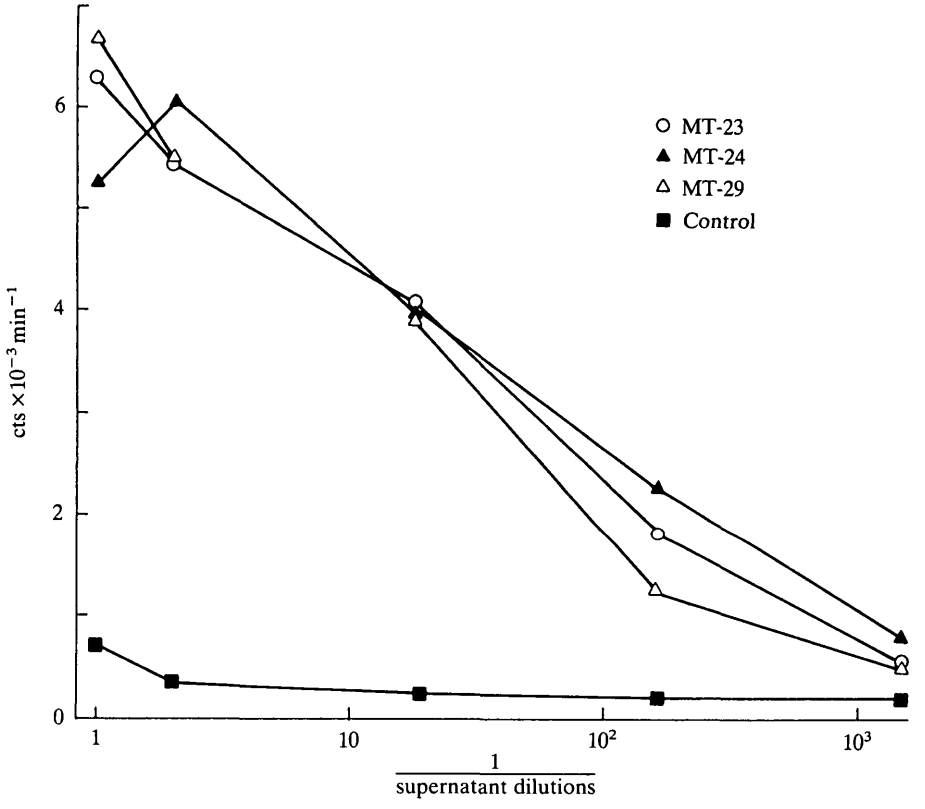

Fig. 4. Binding of MT-23, MT-24 and MT-29 to a suspension of cells from adult mouse testis. A mechanically released preparation of testis cells was incubated with monoclonal antibodies MT-23 (open circle), MT-24 (closed triangle), MT-29 (open triangle) or negative controls HB-11, SP2/0 and serum-free medium. The highest point of any of the negative controls is shown (closed square). The second antibody was ^{125}I-rabbit anti-rat Ig.

beads cross linked to monoclonal antibodies (cf. Fig. 3A,B). Following removal of unbound and some nonspecifically bound proteins from the XT-I-antibody beads by washing with NET buffer, the antigen was eluted by 15 min incubation with 0·5 ml of 3 M-KSCN at room temperature. After a single passage over the XT-I-antibody column, elution and dialysis against NET buffer, the major band in the eluate is the 34 000 M_r XT-1-molecule, accompanied by a small number of minor bands (Fig. 3D). The semipurified XT-1-molecule still reacts with XT-I-antibody, as it can be immunoprecipitated from the dialysed eluate (Fig. 3E) and rebinds to the XT-I-antibody affinity column from which it can be eluted as a single discernible 34×10^3 M_r polypeptide (Fig. 3B).

Additional antibody probing of the XT-1-molecule

Immunoaffinity-enriched XT-1-antigen was used to immunize a rat and prepare monoclonal antibody-producing hybridomas, three of which, MT-23 (IgG_{2a}), MT-24 (IgG_{2b}) and MT-29 (IgG_{2a}), were chosen for further studies due to their

extensive binding to testis cells (Fig. 4). Each of these antibodies efficiently immunoprecipitated the XT-1-molecule. Analysis of these immunoprecipitates from [^{35}S]methionine-labelled germ cells by two-dimensional PAGE indicated that the XT-1-molecule consists of a series of charge isomers. The pI of the major spot was 5·1, with additional isomers forming a tail with decreasing pI (shown in Fig. 5B for MT-29 immunoprecipitate). No polypeptides were specifically precipitated from the culture supernatant (data not shown).

Further characterization of the XT-1-molecule

Immunoprecipitation of the labelled proteins in the effluent and in the bound and eluted fractions from a *Lens culinaris* lectin affinity column was carried out. Comparison of the intensity of XT-1-antigen bands in the two lanes with the total

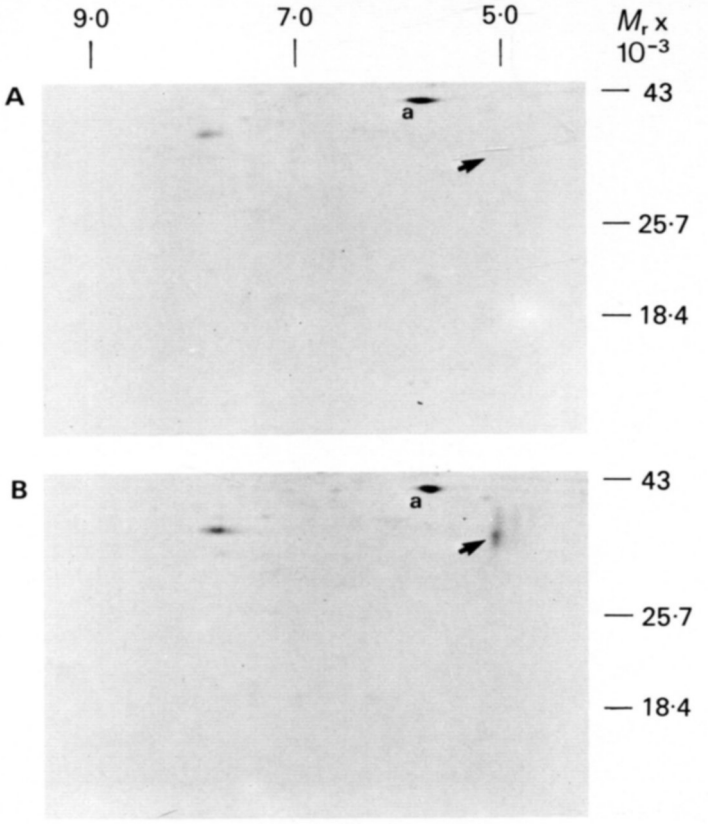

Fig. 5. Two-dimensional electrophoresis of [^{35}S]methionine-labelled cells immunoprecipitated with monoclonal antibodies. Cells from testes of 17-day-old mice were placed in outgrowth culture for 24 h, then labelled by biosynthetic incorporation of [^{35}S]methionine overnight, followed by solubilization and immunoprecipitation with MT-29 (gel B) or HS-19, an unrelated monoclonal antibody (gel A). Horizontal direction is isoelectric focusing from pH 4·5 to 9·5; vertical electrophoresis is 12 % SDS–PAGE. Arrow indicates location of major XT-1-antigen spot. a indicates actin. Bars mark from top to bottom molecular weight markers ovalbumin (43 000), α-chymotrypsinogen (25 700) and β-lactoglobin (18 400).

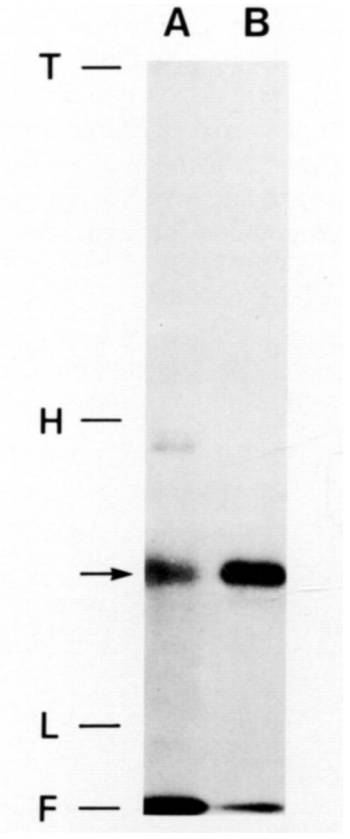

Fig. 6. Immunoprecipitation of XT-1-antigen after binding to affinity column of *Lens culinaris* lectin. The NP40 extract of surface radioiodinated cells was incubated with the *Lens* lectin column, washed with binding buffer and specifically eluted with 10 % glucose. 100 μl of the unbound effluent (containing $5{\cdot}2\times10^7$ cts min^{-1} of iodinated proteins) and of the glucose eluate (containing $6{\cdot}5\times10^4$ cts min^{-1} of iodinated proteins) were immunoprecipitated with XT-I-antibody to recover the XT-1-molecule from these two complex fractions. Following SDS–PAGE, the resulting autoradiographic image demonstrates that XT-1-antigen is enriched relative to total iodinated surface protein in the *Lens* lectin bound (B) *versus* unbound (A) fractions. See legend of Fig. 3 for symbols.

counts of radioiodinated proteins loaded in each lane indicates a substantial enrichment of XT-1-antigen among the proteins that had bound to the lectin column.

The XT-1-antigen appears to be an integral membrane protein and is not significantly extracted from cells by 3 M-KSCN, distilled water or 1 M-urea, but is efficiently solubilized by 0·5 % NP40. This is demonstrable both by radioimmunoassay for antigen remaining on the extracted cells and by immunoprecipitation of the dialysed cell extracts (data not shown).

Epitope groups on XT-1-antigen

The binding of [^{35}S]methionine-labelled XT-I-antibody to testis cells is inhibited by unlabelled MT-24 and by the XT-I-antibody itself but not by monoclonal

antibody MT-23 or MT-29 (Fig. 7A). Similarly, binding of biosynthetically radiolabelled MT-24 is inhibited by the unlabelled homologous monoclonal antibody and by the XT-I-antibody but not by MT-23 or MT-29 (Fig. 7B). The binding

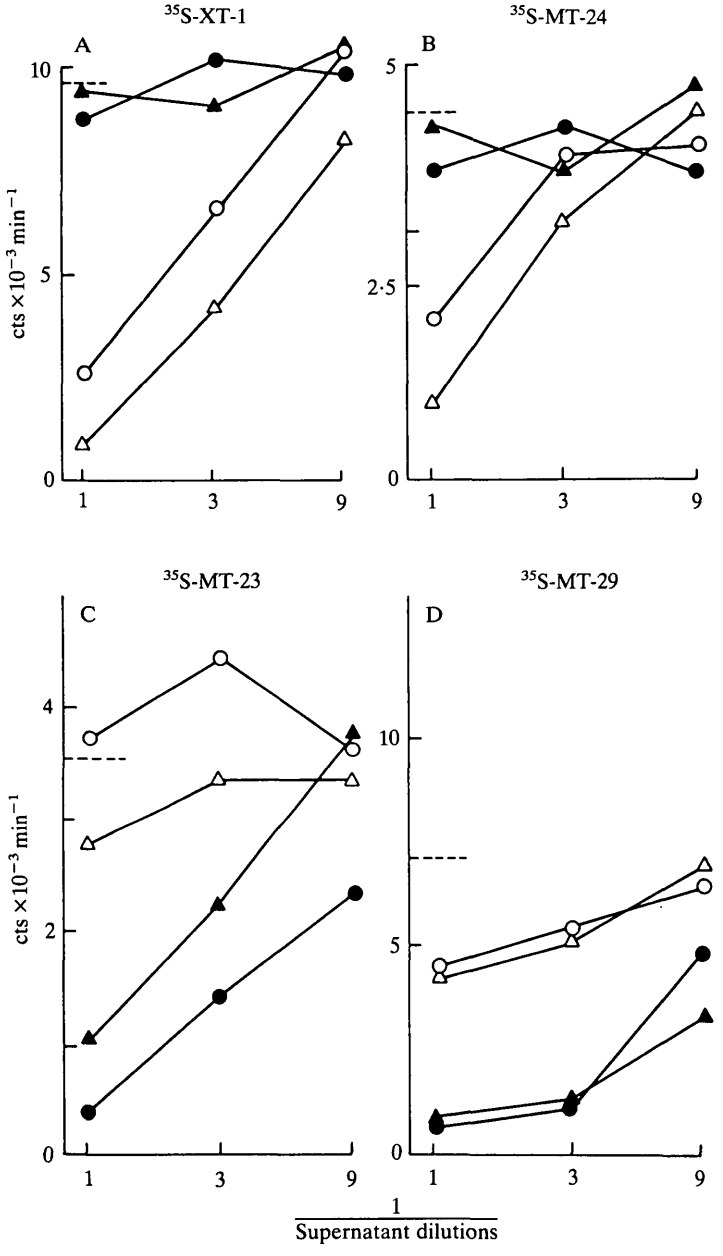

Fig. 7. Inhibition of binding of radiolabelled XT-I-antibody (A), MT-24 (B), MT-23 (C) and MT-29 (D). Dilutions of unlabelled inhibitors, XT-I-antibody (○), MT-23 (●), MT-24 (△), MT-29 (▲) or 0·5% BSA (---), were incubated with target cells, followed by addition of ^{35}S-labelled monoclonal antibodies. Vertical axis is cts min^{-1} bound. Horizontal scale is reciprocal of dilution of unlabelled antibody added.

of labelled MT-23 and MT-29 is efficiently inhibited by both MT-23 and MT-29, but only weakly inhibited by XT-I-antibody and MT-24 (Fig. 7C,D). Thus, two distinct surface regions on the XT-1-molecule are defined by the antibody pairs XT-I/MT-24 and MT-23/MT-29.

DISCUSSION

Specific antibody inhibition of cell–cell or cell–substratum adhesion has been used to identify a large number of adhesion-related surface molecules (for reviews see Damsky *et al.* 1984; Edelman, 1983). In each of these systems, specific antibody blocked cell–cell or cell–substratum adhesion. The antibodies were used in purification of these bound cell surface molecules, thus identifying them as candidates for adhesion molecules. We instead have identified the purified XT-1-molecule as an adhesion-related molecule based on the enhancement of this function upon incubation with anti-XT-1 antibodies (including the monovalent Fab fragment).

The XT-1-molecule was purified by immunoaffinity chromatography and used in immunizations to generate additional monoclonal antibodies so that these might be used to probe molecular function. We have recently shown that three of the monoclonal antibodies (MT-23, MT-24, XT-I and monovalent Fab) binding the XT-1-molecule elicit germ cell–germ cell adhesion, while the fourth monoclonal antibody (MT-29) does not elicit this adhesion (Ho & Bechtol, 1985). As with most other adhesion molecules, it is not known whether the XT-1-molecule after binding monoclonal antibody acts directly or indirectly.

As demonstrated by the ability of each monoclonal antibody to inhibit binding of the other monoclonal antibodies, the three antibodies eliciting cell adhesion bind to at least two distinct epitope groups (XT-I/MT-24 and MT-23) of the XT-1-molecule. In addition, the adhesion-positive antibody MT-23 and the adhesion-negative antibody MT-29 inhibit each other's binding, suggesting either (1) that each alters the tertiary structure of the XT-1-molecule to a configuration not bound by the other, or (2) that they bind sterically close to each other on the XT-1-molecule. In either case this pair of antibodies defines a functionally important aspect of the molecule.

At present the only agents known to elicit germ cell adhesion are the monoclonal antibodies described in the present report. However, two other adhesion-related molecules have been described in testis. A molecule cross reactive with the rat neural adhesion molecule D2 has been demonstrated on late spermatids and residual bodies by immunostaining. The antigen was not detected on spermatocytes or round spermatids (Jorgensen & Moller, 1983). In addition, glycoprotein factor 'clusterin', which causes aggregation of Sertoli cells and erythrocytes from human, rat and sheep, has been found in rete testis fluid (Blaschuk *et al.* 1983), Sertoli cells, rete epithelial cells and some cells of the caput epididymus epithelium, and on the luminal surface and spermatozoa in the caput epididymus

(P. S. Tung & Fritz, 1985). In the ram, clusterin is shed or secreted into the medium of Sertoli cell-enriched cultures and has an apparent relative molecular mass (M_r) of 80×10^3 under nonreducing conditions, a subunit size of approximately $37–40\times10^3$ on reduction and a pI of approximately 3·7 (Blaschuk *et al.* 1983; Blaschuk & Fritz, 1984). Rat clusterin has a subunit size of $34–40\times10^3$ (M_r) with a predominant pI of 7·0 (Blaschuk & Fritz, 1984). In contrast, the XT-1-antigen of mouse is on spermatocytes and spermatids, but is not detectable on or in Sertoli cells or epithelial cells of the reproductive tract (Bechtol, 1984; Bechtol, unpublished observation). XT-1-antigen migrates at 34×10^3 (M_r) both in the presence and absence of reducing agent and is not recoverable from supernatant of tubule outgrowth cultures. It has a series of charge isomers in the pI range 5·1 to approximately 4·5, with the major isomer at 5·1. The differences in cellular distribution and release into culture medium of clusterin and XT-1-antigen suggest that these are distinct molecules. Several other adhesion-related molecules have been identified in other systems, but none has a tissue distribution similar to that of the XT-1-molecule.

Very large, late-pachytene primary spermatocytes were previously shown, by adsorption assays, to have the greatest concentration of XT-1-antigen (Bechtol *et al.* 1979). We quantified XT-1-antigen expression on germ cells isolated from weanling mice when the late pachytene spermatocytes are maximized, and determined there were, on average, 2×10^6 XT-1-molecules per cell. Since this preparation is still a heterogeneous mixture of cells, this figure is most probably an underestimate. Thus, the concentration of XT-1-molecules on these very large cells (average diameter = 16 μm; Romrell *et al.* 1976) is at least $2·5\times10^3$ molecules per μm^2. When compared to a concentration of $1·7\times10^3$ molecules per μm^2 of HLA A, B, and C on peripheral blood B lymphocytes and $0·6\times10^3$ per μm^2 on peripheral blood T lymphocytes (Trucco *et al.* 1980), it becomes apparent that the XT-1-molecule is a prominent membrane component of developing germ cells. Further characterization of XT-1-antigen and the other adhesion-related molecules will be necessary to determine their relationships and relative contributions to adhesion and its regulation at successive stages of development.

The authors would like to thank Ms Marian Butts for excellent technical assistance and Ms Marina Hoffman and Dr Barbara Knowles for reading the manuscript. This work was supported by National Institutes of Health grants GM-23892 and CA-10815, and American Cancer Society grant CD-220. W.C.H. was supported by National Institutes of Health training grant CA-10940.

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(Accepted 2 December 1985)